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Code of conduct for national academies

The national academies of science throughout the world are a mixed bag. Some of them, and certainly the oldest among them, exist more or less by accident. The *Accademia dei Lincei* began in 1603 as a kind of club for cultivated Roman noblemen interested in the doings of those scientists whom they chose to maintain by patronage. The Royal Society in London, also very much a club of enthusiasts, nevertheless won the blessing of Charles II on the strength of its promise that science might help with some of the British government's problems, how most effectively to navigate the oceans for example. Unlike the *Lincei*, the Royal Society has had the good fortune of an uninterrupted history. Elsewhere, national academies have more formal origins. The National Academy of Sciences in the United States, now just over a century old, was set up by the US Congress explicitly to provide advice on scientific and technical questions. Since then, it has become the custom to build in an academy to every new constitution — this, for example, is how the Academy of Sciences of the USSR came formally to be responsible for the administration of a substantial part of the Soviet government's research and development. With the passage of time, the notion has taken hold that national academies, while remaining clubs in the sense that only the membership has a voice in its succession, also have important functions in relation to their national governments.

On the face of things, this relationship is anomalous. What benefits can governments hope to derive from relationships with self-perpetuating societies of scientists? And why should clubs of scientists, having elected each other on the grounds of their achievements in research, then look for a special influence in public affairs for which their scientific work will not have prepared them? These are the questions implicit in Lord Todd's address to the anniversary meeting of the Royal Society last week, at which his five year stint as president of the society came to an end.

Breaking with the customs of gentility that mark these occasions, Todd openly complained that his immediate predecessors had spoiled the relationship between the society and the British government. "From 1950 under three successive presidents", he said, "the society gradually lost influence and drifted away from matters of public policy." The references are to Lord Adrian, Sir Cyril Hinshelwood and Lord Florey, presidents in succession between 1950 and 1965. Todd's analysis is fair, although, in Florey's case, the decision to disengage from government was deliberate. (Instead, Lord Florey tried, without much success, to interest the Royal Society as a whole in matters such as the rate of population growth.) Todd was also right to complain last week that the Royal Society was, as a result, far less able than it might have been to influence the upheavals of the early 1960s — the Robbins expansion of the universities ("ill-considered"), the abolition of the Advisory Council on Scientific Policy, the separation of technology from science and the transfer of the latter to the Ministry of Education (now the Department of Education and Science). But from 1965 until 1970, according to Lord Todd, Lord Blackett's personal commitment to the then new Labour government compromised the independence of the Royal Society and thus further diminished its influence.

The issue here is complicated. Blackett's own influence on the Labour government's policy was immense. Respected as he was by the then Prime Minister, Mr (now Sir) Harold Wilson, Blackett lent his support to some of the favourite policies of the Labour government, for example the Industrial Reconstruction Corporation which acted as midwife to the string of injudicious

industrial mergers of the late 1960s. There can never have been so influential a president. The fact that many of the then Labour government's policies are now recognized to have been mistaken is presumably irrelevant to Todd's implicit definition of how an academy should conduct itself. To be identified with government is to be compromised, even if government policy shows the wisdom of Solomon. But to offer independent advice and even help is not merely common patriotism but in the interests of science and its effective application. This, however, is not an easy balance to strike, if only because of the notorious reluctance of governments to take advice that does not suit them.

Lord Todd's account last week of his own presidency shows what the problems are. He made no secret of his fondness for the Advisory Council on Scientific Policy, which used to publish an annual commentary on the evolution of government policy on subjects as varied as the recruitment of scientific manpower and the conditions (always parlous) of the national scientific libraries. The modern equivalent is the Advisory Council on Applied Research and Development which puts out reports on subjects such as the economic and social consequences of the microprocessor. Todd said last week that the existence of this committee, like the presence of a scientist in the part of the Cabinet Office known popularly as the "think tank", owes something to his own intervention in his role as president. One obvious snag is that, in these respects, the Royal Society has been concerned with mechanisms and not with content and that the arrangements devised fall far short of what Todd himself said last week that he would prefer — the separation of the research councils from the Department of Education and Science and the restoration of the old advisory council. Another, perhaps more serious, difficulty is that the Royal Society has no means by which it can reach a corporate opinion on any substantial issue of public policy. Although, in the past few years, there has been a modest growth of the society's efforts at providing government and parliamentary committees with written evidence on questions such as secondary education or the environment, the process is slow and the outcome is often a muffled recommendation, not a clarion call. The Royal Society would be unable, even if asked, to arrive at anything like a corporate view on, say, government policy on nuclear power stations or whether manufacturers of drugs should in future be liable for side-effects even if there are no known ways of telling in advance what these might be.

To be fair, in this respect even the United States academy is no better off. Congress may commission studies of the hazards of low-level radiation or of climatic change, and the academy will dutifully recruit a panel to do the work without committing each of its members to the conclusions. But neither Congress nor the Administration would welcome the academy's unsolicited opinion on, say, the proper organization of federal research agencies or on the zany procedures followed by Congress in settling the annual research budget. It may be luck for all concerned that the academy would be likely to have as many conflicting opinions on such questions as it has members. Thus even the United States academy, corporately, is not nearly as influential as it likes to think. Its members, especially its president, may often be powers in the land, but almost always on personal grounds — and by personal means, back-slapping and arm-twisting, if it comes to that.

How, then, should academies set out towards Todd's two domestic goals — "to protect and encourage science in all its aspects" and to advise on and help with bringing science and



technology "fully to bear on the formulation of national policy"? The first need is that they should acknowledge their own limitations. They cannot hope too often to bite the hands that feed them without being fed less well. Yet there are some issues on which the academies' unprompted advice would be helpful and even welcome. Both in Britain and in the United States, there is thus a need just now for an independent examination of the relationship between academic research and commercial exploitation. Harvard's problems with genetic manipulation (see *Nature* 4 December) are likely to crop up elsewhere, and in

different fields. The academies are better placed than either universities or governments to say what should be done, but they have been slow to devise an agenda for discussion. (Are they, by any chance, afraid of offending their members?) Similarly, there is an urgent need (and not only in Britain and the United States) for an independently formulated policy on higher education in science, and on the problems of professional people thrown on the scrap-heap before retirement age because of chopping and changing of government policy. If they chose, the academies could be more directly influential than they are.

Further nonsense on product liability

The United States Supreme Court, which does not often make an ass of itself, handed down a decision in October which will already have denied sleep to a host of manufacturers, especially to pharmaceutical manufacturers. The Supreme Court had been asked to rule on the legality of a decision made last March by the Supreme Court of California in the preliminary stages of a suit for damages. Two Californian litigants were suing because of the congenital damage they had suffered because their mothers took diethylstilbestrol during pregnancy. The original claims were faulty in that the plaintiffs did not specify the manufacturers of the diethylstilbestrol from whom they sought damages, but the Californian court helped out by suggesting that any damages eventually awarded might be shared among the suppliers of diethylstilbestrol in proportion to the various manufacturers' shares of the Californian market. The United States Supreme Court has now confirmed that this device is permissible, at least at this early stage. Whether the original suit (which has not yet been heard) will succeed, and will then survive the inevitable circuit of the appeal courts, remains to be seen. But the law on product liability, more stringent in California than elsewhere, is in danger of being made a nonsense. It also promises to be a serious impediment of innovation.

The issues are not complicated, even though the law may be. A manufacturer designs a product, say a mousetrap, sells several copies to the public and makes himself a profit in the process. As time goes on, the mousetraps are used to catch a host of mice, a benefit to the purchasers. But, sooner or later, a clumsy user will impale a finger on a piece of metal or have it broken because the trap goes off prematurely. Who, then, is to blame? In the old days, the law in most countries coincided with commonsense. A person buying a mousetrap intended to injure mice who managed to injure only himself had no cause to sue the mousetrap manufacturer. If he did so, the manufacturer would have been able to plead "contributory negligence" or something of the sort, and the only beneficiaries would have been the lawyers. In future (but already in California), the hapless mousetrap manufacturer will not be able to rely on that defence. First, he must design his mousetrap so that foreseeable accidents cannot take place. Mousetraps that cannot catch fingers will be more expensive and may be less efficient at catching mice, part of the price of product liability. What will keep manufacturers awake at night, however, is the fear that their designers have not thought of every accident. Is there a risk that in emptying the trap of mice, an innocent user may suffer some unsuspected damage? May he contract cancer, for example? For, if that happens, the manufacturer will be liable for damages under the law of strict product liability.

The Supreme Court's decision shows that the vigorous application of this principle offends against natural justice. If all manufacturers of diethylstilbestrol are to be joined in the suit in proportion to their sales revenue in the 1950s from the synthetic hormone, the implication is that no aspect of their behaviour can diminish their share of the liability. Yet some may have been more diligent than others in seeking out unwanted side-effects. Some may have made more profit than others. And some may have gone out of business, leaving the others to carry a greater share of the liability. The reasons why the drug manufacturers are especially

alarmed is clear — identifying unknown side-effects is an open-ended problem, for which reason it seems unfair that virtue is no defence. Strict product liability thus seems inequitable as between individuals. It is too soon to know how many drug manufacturers will turn to making mousetraps because the risks are better known, but strict product liability is a deterrent from doing anything for the first time. The community at large will not in the long run benefit.

This is the manufacturers' case against strict product liability. The other side of the argument is also telling. In the past thirty years, all kinds of manufactured products have turned out to be less safe in use than their salesmen promised. Motor cars have caught fire without warning, or have careered off highways. Aircraft have fallen from the skies unexpectedly, for reasons attributable (with hindsight, easily enough) to faulty design. Electrical appliances have electrocuted their users. And drugs have caused damage as well as bringing benefit, often in the most tragic ways. The wave of product liability legislation springs from the fund of reasonable grievance by innocent consumers against manufacturers who have frequently been slipshod in design or even guilty of telling lies. Manufacturers should not think their troubles will go away spontaneously. In Britain, for example, in the past three years, one royal commission (the Pearson Commission) and two law commissions (one for Scotland, one for England and Wales) have advocated the principle of product liability. How is a line to be drawn between the interests of manufacturers and consumers?

In Europe, the issue is lively just now because of the draft directive on product liability promulgated by the European Commission, and likely to compel national legislation in the next few years. The question is obviously within the interest of the European Commission, for without common legal principles, member states could use product legislation as a way of favouring their own manufacturers. But what should be the common standards? In the past few weeks, the British government (which accepted the principle of strict liability two years ago) has said that it will press the European Commission to allow as a defence by manufacturers the plea that a new product is as safe as the "state of the art" allows. The diethylstilbestrol manufacturers of California would be delighted with such a lifeline in their own case, but it is unlikely to satisfy either the European Commission or European consumers. The snag is that such a defence does not provide manufacturers, either collectively or singly, with an incentive for investing in research and development intended to improve the safety of their products. Indeed, used mischievously, the state of the art defence could become a refuge for the complacent. But is it entirely beyond the lawyers' wit to design a form of words that would allow a manufacturer to defend himself, if something goes wrong with a new product, by pointing to steps prudently taken in the research laboratory to investigate the safety of what he makes? Unless some such device can be found, there is a serious risk that innovation, especially adventurous innovation, will be inhibited. Whatever happens, the drug industry is bound to feel unfairly put upon because its new products have to be tested under government supervision. Should not such a procedure in itself be a defence against a product liability suit?

Congress shares out patent licences

Universities, business, given wider rights

Washington

After several years of intensive lobbying, US universities have persuaded Congress to pass a bill which will enable them to profit directly from the commercial exploitation of any research carried out with federal funds.

The bill was passed hurriedly by both the Senate and the House of Representatives in the last days of the session, and is expected to be signed into law by President Carter before the end of the month.

It makes uniformly applicable across all government agencies an arrangement which universities are already able to make with some departments, giving them the right to license patents arising from the work of federally-sponsored scientists provided that all profits made by the university are ploughed back into teaching or research.

The bill has been strongly advocated by university administrators and patent officers, who have claimed that it will reinforce their efforts to get the results of research onto the marketplace. The bill does not, however, go as far as some would have liked, as it awards patent rights only to small businesses and non-profit institutions, and is therefore not applicable to large companies which receive federal research support.

An alternative version of the bill originally passed by the House of Representatives included comparable coverage of large business. But this provision was fiercely opposed in the Senate, where several members argued that it would result in a "massive give-away" of public resources (*Nature* 6 March 1980).

Rather than prejudice the agreement between the two legislative bodies which is necessary before a bill can become law, members of the House agreed to limit the scope of the bill. They have, however, promised that there will be further attempts to introduce additional legislation covering large companies when the new Congress convenes next year.

In the past, the rights to any patent arising from publicly-funded research have remained the property of the federal government, on the basis that the results of research should be made available to anybody who wants to use them, and that any profits should go towards covering the costs of the original research.

With nobody actively pushing the commercial development of the patents, however, the take-up has been small. Of

the 30,000 patents that have been awarded to the federal government, no more than 5 per cent have been licensed.

In an attempt to give universities a greater incentive to find or create markets for their research results, both the National Science Foundation and the Department of Health, Education and Welfare have recently permitted research institutions to enter into an Institutional Patent Agreement.

Under such arrangements, which have been agreed with more than 30 leading US universities, the institution is allowed to retain patent rights provided that it can present evidence that it will press vigorously to license such patents as it is awarded. (Such an arrangement, for example, has given Harvard University the rights to the results of recombinant DNA research carried out with funds from the National Institutes of Health.)

Even this arrangement has come under fire from some scientists. Two years ago, for example, Professor Joshua Lederberg, now president of Rockefeller University in New York, wrote to a Senate committee suggesting that universities should stay out of the patent business, and suggesting some form of holding institution along the line of Britain's National Research Development Corporation to which patent rights should be allocated.

But support for expanding the principle to cover all federal research came both from the universities, who see royalties as a potentially fruitful source of additional income, and from the Carter Administration, which had made patent law reform a major recommendation in last

year's domestic policy review of industrial innovation.

Under the terms of the new law, all federal research agreements with small businesses and non-profit institutions will carry a clause stating that the institution carrying out the research will own the 17-year rights to any patents provided certain conditions are met.

In the case of universities, all licensing agreements must be approved by the funding agency; exclusive licences can only be awarded to large companies for between 5 and 8 years, after which they must be made generally available; royalties must be shared with the inventor; and the balance of royalties or other income made after the deduction of reasonable expenses must be used to support scientific research or education.

In addition, the federal government retains "march-in" rights to direct the university to award licences if it feels these are being inadequately handled. Any products or processes arising from the patent for the domestic market must be produced "substantially" in the United States. The earlier requirement that universities should share their profits with the government has, however, been dropped.

The bill also included a separate section extending the copyright laws to cover computer software. At present, software compilers have no way of protecting their programs from being copied and used without their permission. Now a software program which has been copied can be used only where permission has been granted, or for archival purposes. **David Dickson**

Stanford and UCLA plasmid patent

Washington

A patent was awarded last week covering some of the fundamental techniques used in recombinant DNA research and developed by Dr Herbert Boyer of the University of California, San Francisco, and Dr Stanley Cohen of Stanford University.

The patent covers a process developed in the early 1970s for inserting foreign genetic material into a bacterial plasmid. The method has since become widely used by almost all of the companies engaged in recombinant DNA research. These companies will now have to obtain a licence to use the techniques.

The patent, covering the United States only, was issued to Dr Boyer and Dr Cohen as inventors, but they have already agreed to assign the rights and royalties that may ensue to their respective institutions. Mr Niels Reimers, director of Stanford's technology licensing office, said, however, that any royalties demanded would be

"reasonable", and stressed that, at least initially, it was not expected to make a large amount of money from the patent. Also the techniques developed by Boyer and Cohen can still be used freely by scientists for research purposes.

The question of payment is sensitive. If the patent turns out to be lucrative for the two universities, it could be challenged on the grounds that the patent application did not acknowledge the contribution which scientists other than the two named inventors had made to the techniques described (*Nature*, 3 April 1980).

Several scientists said last week that they were waiting to read the details of the patent — and to see what licence fee Stanford and the University of California, San Francisco were likely to demand from private companies — before deciding whether to take any further action.

The terms of the patent, No.4237224, are intentionally broad. They cover "a method for replicating a biologically

functional DNA'', and describe the various steps used to cleave viral or circular plasmid DNA, insert a separate DNA fragment, and grow up and separate unicellular organisms containing the altered DNA.

Still awaited is a decision from the Patent and Trademark Office on a second application made by the two universities on behalf of Dr Cohen and Dr Boyer, which covers any organism produced with the techniques covered in the first patent.

Mr Reimers said last week that, from the universities' point of view, this was likely to be the more important patent, since without its protection companies could manufacture products abroad using the recombinant DNA techniques, and subsequently sell them in the United States without having to pay royalties.

The Patent Office's decision on the second application is expected within a year. All such patent applications covering microorganisms had previously been held up pending a ruling from the Supreme Court, which decided in the summer that there is nothing in existing patent law which denies microorganisms protection.

Meanwhile, the University of California has filed a suit against the pharmaceutical company Hoffman LaRoche and the San Francisco firm Genentech, claiming that the two companies must pay damages to the university for the use of a cell line produced by university scientists which the companies have been developing as a potential commercial source of interferon.

The scientists at the University of California, Los Angeles who produced the cell line claim that it was passed to Hoffman LaRoche without their permission. But in the counter-claim Hoffman LaRoche is arguing that there were no conditions attached to the cell line when it was obtained indirectly through a researcher at the National Cancer Institute, and that the company therefore has no obligation to the university.

David Dickson

Genetic engineering

Hormone growth

Genentech, the California-based biotechnology company, will begin clinical trials of its latest genetically-engineered product, human growth hormone (HGH), in London this January. But Genentech's industrial partners in the venture, Kabi Vitrum AG of Sweden, are somewhat ambivalent about the development.

Kabi Vitrum is the major world producer of HGH, made at present from cadaver pituitaries. The hormone is used to treat HGH-deficient children, reckoned to be some 7-10 per million of population. But the nature of the source naturally limits production, and Kabi estimates the true market to be three times the present supply; so the firm searched for other sources. Genetic engineering was an obvious possibility, as HGH is a small

peptide, about the size of insulin.

So in 1978 Kabi asked Genentech to produce a strain of *Escherichia coli* containing the HGH gene. Under the contract, Kabi would have sole world production rights, except in the United States and Canada, where they would be shared with Genentech. But Genentech was successful sooner than Kabi expected, and further surprised the firm by making rapid preparations for commercial production. Genentech had been expected to stick to research.

Now Genentech is well ahead with pilot tests in 700-litre fermenters, while Kabi has been restricted to 10 litres by Swedish limits on scale-up of genetic engineering experiments; and Genentech plans clinical trials at Great Ormond Street Hospital for Sick Children starting in January 1981. Kabi, meanwhile, would wish to be more cautious. "We do not know about Genentech's toxicological testing" said Dr Bengt Karlsson, Kabi's managing director, "but we would not wish to put the product on clinical trial for at least six months". Nor is Kabi's caution due to lack of material, for they have access to Genentech's supply.

However, in London, where Dr James Tanner at Great Ormond Street will conduct the trial, it is felt that Genentech's toxicity testing has been quite sufficient. There have been plenty of animal and monkey tests, says Dr Tanner, and the UK Department of Health has passed the material for clinical trials. The Genentech HGH will be given to 20 patients for a year, 10 of them first treatments, and the other 10 already three years into a course of pituitary HGH. The main contaminant will be 1-2 per cent of bacterial protein; and the danger is that HGH-like proteins in this material may induce antibody formation to true HGH.

Dr Tanner welcomes the new source of the hormone. In the United Kingdom there are 100 new cases a year of HGH-deficiency, and just enough pituitary HGH to go round (produced from 60,000 cadavers a year). But it is always "touch and go" each year whether sufficient cadavers will be made available. Moreover, if there were more HGH around, it could be tried out on more marginal cases of delayed growth, or slow bone healing after fractures.

Ultimately, Genentech will not be the source of HGH in Britain. Kabi announced last week that it has concluded a deal with the Department of Health for the Centre for Applied Microbiological Research at Porton Down to conduct scale-up trials on the Genentech bacterium. In exchange, Kabi will offer the product at a preferential price to the Department of Health, and assist Porton with its present production of HGH from pituitaries. (Kabi believes it has a more efficient extraction system.)

The centre is seeking permission from the Genetic Manipulation Advisory Group to ferment the engineered *E. coli* in

400-litre vats; and to satisfy GMAG, it must show that the organism is killed in the closed fermenter before the material is extracted.

Despite early misgivings, the Department of Health has begun to encourage Porton to become involved with industrial applications of the recombinant DNA technique — Unfortunately nothing has yet come forward in the United Kingdom that fits the bill. Dr Peter Sutton, director of Porton, is delighted at the deal with Kabi: "It means we can get our feet wet" with commercial scale genetic engineering, he said last week.

Robert Walgate

US Administration

Reagan's men?

Washington

President-elect Ronald Reagan's transition staff is sifting through the list of candidates for top-level positions in the new Administration, and the names of the first cabinet appointments should be announced this week. Potential choices have been widely discussed in the press — often names intentionally leaked to gauge public reaction — so few major surprises are expected.

Top candidate for the renamed Department of Health and Human Services (DHHS), responsible for the biomedical research budget of the National Institutes of Health (NIH), is Senator Richard Schweiker, Mr Reagan's running mate in his bid for the 1976 Republican nomination.

Mr Schweiker gave up his Senate seat earlier this year to work for Mr Reagan's election. In the Senate he was an active member of the Labor Committee's health and scientific research subcommittee, and as ranking minority member often supported the initiatives of the committee's present chairman, Senator Edward Kennedy.

Mr Schweiker is "definitely pro-science", one NIH official said last week, although adding that he had received some criticism for inserting in the institutes' funding legislation a clause that requires special arrangements for supporting research in diabetes and digestive diseases — the type of constraint that NIH prefers to work without.

The appointment would be less popular with labour unions, since Mr Schweiker is the sponsor of a bill designed to restrict the activities of the Occupational Safety and Health Administration.

As DHHS Secretary, Mr Schweiker would be responsible for the budget and activities of the National Institute of Occupational Safety and Health, part of the Center for Disease Control.

A predecessor in that post, Mr Caspar Weinberger, is widely tipped as next Secretary of Defense. He was director of the Office of Management and Budget under President Nixon, where his financial

stringency earned him the nickname "Cap the Knife". He was later promoted to Secretary of the then-named Department of Health, Education and Welfare at a period when biomedical research was becoming dominated by a congressional "disease of the month" approach.

Various names are being discussed for the position of Under-Secretary of Defense for Research and Technology, responsible for the Pentagon's massive research budget. They include Mr William van Cleave, at present head of Mr Reagan's defence transition team, and Mr Benjamin T. Plymale of the Boeing Corporation, who was the source of controversy last year when his security clearance was temporarily revoked.

No clear candidate has yet emerged to head the Department of Energy. One suggestion, Mr Michel Halbouty, a Houston oilman and geologist who was Reagan's chief energy strategist during the campaign, is being opposed by some influential Republicans because of his lack of government experience. Others have opposed the nomination of Mr Frank Zarb, a top energy official in the Nixon and Ford administrations, because of his involvement in setting up the present system of price controls on crude oil and gasoline. Two possible contenders are Dr John Sununu, professor of engineering at Tufts University, Massachusetts, and Representative Clarence Brown of Ohio.

Appointments at a lower level, including the heads of independent agencies such as the National Aeronautics and Space Administration, are not likely to be announced until the main cabinet posts have been filled.

In the science field, these appointments will also depend on the report of the science and technology transition team under Dr Simon Ramo of TRW and Dr Art Bueche of General Electric.

Dr William A. Nierenberg, director of the Scripps Institute of Oceanography, is widely mentioned as possible director of the Office of Science and Technology Policy (OSTP), as is Dr Guyford Stever, ex-director of the National Science Foundation, who held the OSTP job for a few months at the end of the Ford administration.

At the National Science Foundation (NSF) itself, the Reagan administration seems unlikely to overturn the appointment of Dr John Slaughter as director. Dr Slaughter was sworn in two weeks ago, and that the emphasis that he is keen to put on the development of engineering and applied research should match Republican goals for science.

Finally, the appointment of Dr Frank Press, the present director of OSTP and President Carter's Science Advisor, was assured as the next president of the National Academy of Sciences when nominations for the post closed last Monday without any other names having been put forward.

David Dickson

Soviet plans

Science on tap

Soviet science is to be geared even more closely to the needs of the economy, according to the guidelines for the 11th Five Year Plan, published last week. The plan calls for a substantial reduction in the time taken to disseminate research results, strengthening of the links between research and production, better coordination between scientific establishments and an improved basis for scientific planning.

Individual research priorities specified by the guidelines range from the immediately practical (the improvement of computer technology and software) to the long-term (creation of the bases for thermonuclear power engineering), and from the further conquest of space to greater environmental protection and economic utilization of the biosphere. Biotechnology to produce new compounds with tailor-made properties, particle physics and immunology all receive special mention.

At this stage of planning, however, no specific targets are mentioned, nor is the financing of science discussed. The emphasis on closer links between science and industry, however, and the statement that ministries and departments are to bear increased responsibility for industrial research may have some financial implications. Their responsibility will presumably also include the planning of research in institutes under their control. One of the main complaints of Soviet scientists in recent years has been the inflexibility of research plans once approved. The new guidelines, however, urge that the direction of research and development should be "determined in good time . . . and changed to meet the demands of the scientific-technological revolution".

All this, however, depends on an overall increase of labour productivity. In industry, this increase is specified as 23–25 per cent, which is to account for more than 90 per cent of the increase in output. For the scientists no such target is set, perhaps because the recent "press debate" in *Literaturnaya Gazeta* has revealed only too clearly how much scientists resent having their intellectual performance monitored.

Vera Rich

DNA guidelines

Bowing out

The US National Institutes of Health (NIH) are facing a virtual revolt from local Institutional Biosafety Committees (IBCs) over whether there is still a need for strict surveillance of research using recombinant DNA techniques.

At a meeting in Washington organized by the National Institute of Allergy and Infectious Diseases, the predominant view

of the chairpersons and representatives from more than 150 IBCs throughout the country was that the prime role of the IBCs has become largely a public relations exercise.

Few of those attending the meeting were prepared to accept that recombinant DNA research presented any greater health or environmental hazard than work with unaltered organisms not covered by the NIH guidelines.

Many complained of the amount of paperwork they are required to carry out, particularly in the light of recent revisions of the guidelines, which have shifted most of the responsibility for reviewing research protocols from the NIH's Office of Recombinant DNA Activities to the local level.

The Washington meeting had originally been called for IBC chairpersons to discuss how their committees were operating. But the main focus of the two-day meeting rapidly became whether the IBCs — or even specific regulations covering recombinant DNA research — were any longer needed in their present form.

According to one NIH official, the mood of the meeting was that the amount of time that IBCs put into DNA issues was out of proportion to all sorts of other bio-hazards.

One recommendation being forwarded to next month's meeting of the NIH's Recombinant DNA Advisory Committee is that all experiments using the disabled K12 strain of the bacterium *Escherichia coli*, or the yeast *Saccharomyces cerevisiae*, as host-vector systems should be totally exempt from the guidelines.

In the case of *E. coli*, the same suggestion was made last year, but the advisory committee then recommended — and NIH director Dr Donald Fredrickson agreed — that although prior approval was no longer necessary for such experiments, the requirement that the experiments be carried out under P1 physical containment conditions should remain.

Members of biosafety committees also complained about the additional paperwork resulting from NIH's requirement that, although details of all approved experiments no longer have to be registered, they must keep detailed records of all recombinant DNA work carried out in their institutions.

The latter requirement was partly the result of a survey at Stanford University in California which showed a discrepancy between the rate at which different committees required experiments to be reclassified, possibly indicating that some were interpreting the guidelines more strictly than others.

But the IBC members balked at yet more paperwork.

A straw vote taken during the final plenary session of the meeting revealed little support for the proposal that NIH should keep a record of all recombinant DNA research carried out under the guide-

lines, a small majority for recording research carried out under P2 containment conditions and above, and a larger majority for keeping a record merely of all research in P3 and P4 containment conditions, the two strictest categories.

Reflecting their general belief that recombinant DNA research no longer represents a greater hazard than ordinary research with microorganisms, many committee members were sceptical about the value of a broad study of the effectiveness of IBCs which NIH is now preparing.

If the committees had any value, it was felt, it had been in calming public fears about the health implications of such research. Mr Ray Thornton, for example, recently appointed chairman of the advisory committee, said that the careful supervision of experiments had been largely responsible for the general development of public confidence.

Other speakers suggested that, even if no extra hazards had been identified, the public discussion raised by initial fears had helped to generate a consciousness about the need to watch for biohazards in general.

David Dickson

Yugoslavia now

Supek's worry

Yugoslavia could shortly face economic collapse if the planners fail to make proper use of the country's scientific personnel. This is the opinion of Dr Ivan Supek, the Yugoslav physicist and philosopher, in London this week for a Pugwash meeting.

Yugoslav scientific and academic life, says Dr Supek, has almost completely lost the impetus of 20 years ago. The financing of basic research is hampered by a bureaucratic system which allegedly subordinates research to consumer control. But reliance on foreign licences (usually purchased when already obsolescent) means that the technological base required by Yugoslav industry and agriculture is either inappropriate or altogether absent.

The chief factor in the decline, according to Dr Supek, is excessive party and state control over science. After the hardliners' coup of 1971, the universities lost much of their autonomy, including the right to elect their own deputies to parliament. (Dr Supek himself was a non-party deputy from 1963 to 1967.)

At the same time, the university structure was decentralized. The University of Croatia, for example, was divided into four separate universities (Zagreb, Split, Rijeka and Osijek) and the individual faculties, rather than the university as a whole, became the basis of planning and financing. Frequently, said Dr Supek, decision-making fell into the hands of party members with no particular academic background.

Under these arrangements, "censorship by budget" was made easier. Among the

victims of this process was the *Encyclopedia Moderna*, a philosophy of science journal edited by Dr Supek himself.

Dr Supek stresses that it is not the "self-management" process — Yugoslavia's special contribution to socialism — which is at fault. If the current trend towards bureaucratic centralism could be reversed, he said, and "self-management" restored to scientists, both basic and applied research would benefit. At present, however, self-management is simply a slogan.

Such official duplicity, said Dr Supek, is nothing new in Yugoslav science. In 1956, when Yugoslavia began a nuclear research programme, Dr Supek, as director of the prestigious Rudjer Boskovic Physics Research Institute, found himself *ex officio* on the country's atomic energy commission. Although the programme had, officially, a purely scientific and peaceful orientation, its members included the Minister of Defence Ivan Gosrjak and



Supek (right) and defence minister, 1956

Minister of Internal Affairs Alexander Rankovic. Their presence made Dr Supek extremely sceptical of the true aim of the programme, and made him a fervent opponent not only of nuclear weapons but of all applications of nuclear energy.

The duplicity, which, in Dr Supek's words, is allowing self-management to be killed in the name of self-management, will be a major obstacle to any move by the scientists to regain their pre-1971 position. The recent ban of the proposed cultural and sociological journal *Javnost* was justified by the Belgrade authorities on the grounds that the journal was meant to be a front for a would-be cultural elite. Dr Supek, however, is strongly opposed to elitism, and would claim for science only that right of self-government which is constitutionally guaranteed to all Yugoslav workers. The Party hardliners, however, are not prepared to yield without a struggle. Recently agronomists working on the forthcoming five year plan proposed that, for modern farming methods to be introduced, the maximum peasant holding should be increased from 10 to 50 hectares. But in spite of the deteriorating state of Yugoslav agriculture, the proposal was rejected as liable to cause class conflict.

Vera Rich

Agricultural research

Ministry at top

An impending change in the relationship between the Agricultural Research Council and the Ministry of Agriculture, Fisheries and Food (MAFF) now seems likely. Most probably, the council will in future be more directly subject to the ministry. In this respect, it is likely to be worse off than the Medical Research Council, which in October reached an arrangement with its chief sponsoring department of government, the Department of Health and Social Security, that some £12 million of "Rothschild money" should be transferred back to its own annual budget.

Change has been in the air since the publication of a report of the Public Accounts Committee in July 1979 which suggested that the government should consider transferring a further slice of the ARC budget to the agriculture ministry. The underlying principle is that put forward in 1971 by Lord Rothschild, who advocated giving control of research budgets to the chief users of the results of research — the ministerial "customers". At present, some 40 per cent of the research council's spending derives from the ministry, and the Public Accounts Committee was asking why the balance should not be shifted further.

For the past year, a committee under the chairmanship of Sir Brian Hayes, permanent secretary at the ministry, has been trying to decide what should be done. The alternative to a further transfer of funds is a more direct influence by the ministry on the policy of the council. Although a decision has not yet been reached, the second course is the more likely. Either way, the council is unlikely to be overjoyed.

Like many government-supported institutions, the council (ARC) has been hard-pressed to operate within its cash limits during the financial year 1979–80. Nevertheless, by the end of that year, says its annual report published last week, it had managed to plan a reasonable research programme for 1980 and beyond by concentrating its efforts on high priority research.

The council's choice of priorities was effectively made by MAFF which has cut the amount of research it is prepared to buy in some of the ARC's research institutes while increasing it in others. MAFF currently pays for about half of the work conducted in ARC institutes under the Rothschild customer-contractor principle.

MAFF is particularly keen to encourage food research, interest which has proved lucky for the ARC's Meat Research Institute whose grant of £370,000 from the Meat and Livestock Commission was cut last September. MAFF has stepped in to make up some of the loss. It has also increased its contribution to the budget of the Food Research Institute in Norwich,

the extra support going mainly to research on biopolymers, nutrition and biotechnology. This new emphasis coincides with a reorganization of the Food Research Institute and the setting up of a joint ARC-Medical Research Council working party on food research policy.

Irrespective of MAFF, ARC has been increasing support for research in priority areas, in particular by introducing a new scheme for supporting work in universities outside the normal research grant system. ARC research groups in photosynthesis have been set up at the universities of Leeds and Sheffield and at Imperial College, London; and at the University of Bristol a group has been established in the neurobiology of animal behaviour and reproduction. The ARC's plans for the forthcoming year include the establishment with the Medical Research Council of a new unit at Edinburgh on neuropathogenesis.

Judy Redfearn

British dentists

Broader, better

The proposal that dentists newly qualified from British dental schools should not be allowed to practise without first spending a year in supervised practice is the chief recommendation of an inquiry into dental education, commissioned by the Nuffield Foundation and published earlier this week. The inquiry was directed by Sir Gordon Wolstenholme and supervised by a steering committee under Professor T. C. Thomas, previously vice-chancellor of the University of Liverpool.

British dentistry, long saddled with an international reputation not very different from that of the British motor car industry, is offered a cheerful future in the report (to be had from the Nuffield Foundation at £4.50). The incidence of caries among children is decreasing, dramatically so in cities such as Birmingham where there has been fluoridation of water supplies for some time. In the ten years from 1968, the proportion of the British population entirely without teeth (described in the report as "the edentulous state") decreased from 36 per cent to 29 per cent.

Part of the stimulus for the inquiry was the recognition that dentists now being trained would still be in practice when the state of British dental health has been further transformed, so that the pattern of practice will have changed, and when novel dental technology will be available.

The theme of the inquiry's report is that dentists should be trained for flexibility. The dental schools are told that their present output of professionals is likely to be sufficient for the future, but that British dentists should rely more than is their present habit on ancillaries, especially hygienists and therapists, all of whom are in need of more explicit training and career structures.

On the curriculum of the dental schools,

the report argues for greater attention to basic science and the early introduction of students to clinical work. Present dental courses are too narrowly vocational, and should be broadened but not lengthened (at present training lasts four or five years). Instead, the argument goes, there should be a pre-registration year.

But where? Newly qualified physicians spend their pre-registration years in hospitals, but dentists have no comparable places of supervised work. The committee suggests a system of supervision in practice organized by the dental schools and based on the Community Dental Service or on the dental services provided for the forces. It also wants to see a period of vocational training in the first two years of a dentist's regular practice and more opportunities for in-service training than there are at present.

The committee of inquiry also asks that steps should be taken to increase the present scale of dental research in Britain, including the setting up of a national research centre. One obvious present difficulty is that most dental research is at present undertaken on short-term research grants.

The committee neatly skirts around the most contentious problems in British dentistry — how should dentists be paid? It echoes the general discontent (shared by some dentists and most National Health Service patients) that the present system of piecework payments is unsatisfactory, but judiciously recommends that professional organizations "should seek a system of payment . . . other than that existing at present".

Israeli science

Crisis at Weizmann

Financial crisis threatens the "very existence" of the Weizmann Institute of Science, Israel's private and principal research institution — or at least that is what Weizmann president, Professor Michael Sela, told the governing body recently. But according to the Weizmann Institute Foundation in London, which helps to raise money in the United Kingdom for the institute in Israel, the Weizmann finances have never been better.

Somewhere between these two statements lies the true position — that the Israeli government, which provides 45 per cent of the institute's running costs, has announced government spending cuts which will reduce its contribution to the Weizmann Institute by 7½ per cent (in real terms) compared with last year; and that Professor Sela was painting a grim picture to drum up support from the Weizmann's foreign friends, who are strongly represented on the governing body.

Foreign endowments and bequests from Europe as well as the United States contribute around 20 per cent of the Weizmann's funds. While these contri-

butions have been increasing, they have not kept up with inflation outside Israel — except for the United Kingdom, whose donation has increased by two-thirds in the past two years. This is due to the encouragement of Lord Sieff, chairman of the major British chain store Marks and Spencer, and also chairman of the Weizmann Institute board of governors.

The net result is that the Weizmann budget will be static this year (October 1980 to September 1981) in dollar terms at about \$30 million, whereas institute officials would have liked to see a small growth to take into account inflation abroad (where scientific equipment, for example, must mostly be bought).

Nevertheless, in 1980 funds were sufficient to establish, on a scientific staff of 300, five new "career development



Sela, crying wolf?

chairs", as an exercise to introduce new talent to the campus, and 10 professorial chairs. Money for these came mostly from the United States, but also from France and South Africa. Two new research centres were also established within the institute: the K. B. Weissman Institute of Physical Sciences and the Melvyn A. Dobrin Centre of Plant Research. Moreover, the Yeda Research and Development Company, which acts as a link between the institute and industry, increased its turnover to \$4.5 million in 1980, compared with \$1 million three years ago. (One recent venture, Inter-Yeda, manufactures human fibroblast interferon for use in clinical trials.) The government cuts must therefore be seen in the context of a recent very healthy spending pattern at the Weizmann.

Nevertheless, 70 per cent of the institute's budget is spent on salaries; so the effect of a cut in total budget is magnified when compared with the 30 per cent the institute has to spend on books, equipment and fuel. Bequests on the whole do not provide these items; so cuts are, in the end, having to be made in these financially marginal but scientifically significant areas. Because a potential donor is not likely to want his name on a batch of test tubes or even a spectrophotometer, the Weizmann may find it more difficult to raise this kind of small change abroad.

Robert Walgate

CORRESPONDENCE

Medical schools

SIR — Although your editorial of 27th November 1980 on the reorganization of London Medical Schools has some hard things to say about London University in general and some of its senior members in particular, for these your writer may perhaps be forgiven, since they are largely matters of opinion. Less forgivable, however, are the errors of fact that spice the article. I wish to draw attention to only two of these. You refer to "two preclinical schools (at King's College and Westminster Hospital)". The Westminster Medical School has no preclinical departments of its own: King's College is a preclinical school for both King's College Hospital Medical School and the Westminster Medical School. More important, you continue "... the effect of the Senate's decision is that the preclinical schools at King's College and Westminster Hospital (sic) should be closed ...". The Senate's decision implies nothing of the sort: *all* preclinical schools of the University, including that at King's College, are to be reinvestigated in the next few months. Which school, if any, is to be recommended for closure is still a matter of speculation, even for members of the new Working Party. The article does contain one germ of good sense, where it refers to planning. If there is or ever has been a plan, would that the hewers of wood and drawers of water could be told it.

K.E. WEBSTER

Department of Anatomy,
University of London, King's College,
London WC2, UK

Badgers and TB

SIR — Following public criticism of the policy of the Ministry of Agriculture, Fisheries and Food (MAFF) for dealing with badgers infected with tuberculosis (TB) the Minister of Agriculture asked Lord Zuckerman to take an objective look at the relationship between badgers, cattle and bovine TB, and to advise on how the problem should be tackled in the future¹. This is a complex question. The Mammal Society agrees with Lord Zuckerman's basic conclusion that the badger is a major reservoir of bovine TB in certain limited areas of South West England, and hence is a potential danger to the cattle in those areas. However, we feel that the report gives a biased interpretation of the evidence, and that many of Lord Zuckerman's conclusions were not justified from the data presented in the report. We would like to correct some of the factually misleading statements.

During the period of the moratorium on gassing, the percentage incidence of TB in badgers from Gloucestershire and Avon increased, an observation that Lord Zuckerman took to imply that the disease has "spread". The fact that the percentage incidence declined slightly in Cornwall during the same period was overlooked (p. 63). Also, Lord Zuckerman's statement (p. 40) that at

least one in five to one in ten badgers in affected areas is now infected with TB is misleading. The incidence quoted relates largely to samples taken in the vicinity of TB outbreaks in cattle, and there is no reason to believe that such high levels of TB are to be found except in very small pockets of infection. Certainly there is no scientific evidence to justify Lord Zuckerman describing badgers in the South West as a "highly-infected population" spreading TB to badgers in other areas.

Since TB in badgers appears to be confined largely to limited areas of the South West, and since the evidence to suggest that the disease is spreading is equivocal, we strongly contest Lord Zuckerman's view that bovine TB is a major hazard to the survival of the badger.

Lord Zuckerman stated (p. 27) that the gassing of badgers was accompanied by a decline in the number of reactors in the cattle herds concerned, and that the two events were related. However, the rate of decline in the incidence of TB in badgers in the South West was paralleled by a decline in the incidence of reactors in cattle herds not only in the South West but also in the rest of England. The timing and rate of decline were similar in all three samples. Lord Zuckerman offered no explanation for this. One possible interpretation is that the incidence of TB in both badgers and cattle underwent a decline throughout the country, and that the badger gassing campaign had little significant effect on the overall timing or rate of decline, though gassing may have affected the situation locally.

Lord Zuckerman concluded that the appearance of the disease in cattle in the South West reflected a high local prevalence of TB in badgers (p. 41). This is manifestly untrue; in Cornwall only 15 per cent (51/340) of herd infections were definitely attributable to badgers (p. 57).

Lord Zuckerman stated that "population density is a major factor in the spread of TB". Certainly it is probable that population density is a factor in the spread of disease, but other more subtle factors may be significant. Badger and cattle densities² in parts of Dorset and Somerset are as high or higher than in Gloucestershire, Avon and Wiltshire, yet the incidence of TB is very much lower. Why? Also, the presence of infected badgers does not always result in reactors in nearby cattle herds (p. 21). Why?

Lord Zuckerman speculated that badgers all over the country once suffered and died from TB, and that the prevalence of TB in badgers had declined in parallel with the decrease in the incidence of TB in humans and cattle (p. 41). But since badgers are a self-perpetuating reservoir of tuberculosis (p. 95), it is difficult to see why, when the incidence of TB was reduced in cattle, it should also have declined in the badger. This is particularly inexplicable in areas such as parts of South East England and North Wales, where badger densities are comparable to those in the South West (p. 68). In the absence of any evidence to support Lord Zuckerman's view, it seems more logical to speculate that TB was never as prevalent in badgers in the rest of England. So what factors make the situation so different in parts of the South West?

There are many anomalies in the data presented in the report; we have only mentioned a few. Clearly many issues remain unresolved, and there are likely to be many subtle factors involved in the process of transmission of TB from badgers to cattle which are not yet understood. We believe that the continuation of the badger gassing campaign should only be regarded as a short-term expedient. Lord Zuckerman accepted that eradication of TB in badgers is probably impossible (p. 27). In that case, there is every reason to suppose that if/once gassing operations cease, and the badger population builds up again, the incidence of TB in the badger will also increase. In order to achieve an acceptable long-term solution we believe that it is imperative that further research is undertaken to (1) explain some of the many anomalies in the data available and (2) understand the factors involved in the transmission of TB from badgers to cattle.

STEPHEN HARRIS

The Mammal Society,
Reading, Berks, UK

1. Zuckerman, Lord. *Badgers, Cattle and Tuberculosis: Report to The Right Honourable Peter Walker, MBE, MP* (HMSO, London, 1980).
2. Ministry of Agriculture, Fisheries and Food — *Agricultural Returns — England and Wales, Regions and Counties — Final Results of the June 1979 Census* (HMSO, London, 1980).

Court feasibility

SIR — Your United States election scoreboard of pluses and minuses (*Nature* 13 November, p. 107) scores the President-elect's statement that he would explore the feasibility of a "science court" as a "plus". This seems only justifiable if one regards a statement that feasibility will be explored as preferable to one that the concept will be implemented. At the risk of making a hackneyed point that is, however, evidently unfamiliar to your reporter, it cannot be too strongly emphasized that the procedures of science and a court of law are necessarily different. To take only the best known example: the verdict of a court of law can take only one of two values — guilty or not guilty. In science, there are no probabilities that are equal to zero or one.

C.R.B. JOYCE

Ciba-Geigy AG,
Basle, Switzerland

Plusses and losses

In the article by David Dickson on the likely consequences of the election of Mr Ronald Regan as President of the United States, published on 13 November, the likely changes in US science policy were described as "Plusses" and "Minuses", not as "Winners" and "Losers" as in the original article. The complaints of Messrs Noble, Klimowsky, Vollamy, Price and Joyce (*Nature* 27 November) therefore lie against the London office and not against Mr Dickson.

EDITOR, *Nature*

NEWS AND VIEWS

Conflict and commitment in neural development

from Miranda Robertson

TWO controversial questions dominate research on the development of the nervous system. The first, which has the longer history of controversy, is that of how growing nerve fibres make specific connections with their targets. The second is that of the extent to which neurotransmitter specificity is induced by the micro-environment of the nerve cell or its axon during development. It was clear from the collision of views at the annual Symposium of the British Society for Developmental Biology* that both issues remain contentious, but for different reasons.

Specification of connections

The principal difficulty with research on the specificity of connections (or Sperry-specificity, as one participant nicknamed it) is that the complex results of manipulating an embryo have too often been interpreted on the basis of too limited an investigation. One of Sperry's own first contributions to the field was to reinvestigate in detail the effects on the motor performance of adult animals of crossing their motor nerves, which was said at the time to lead to central respecification and the reestablishment of normal movement. Sperry showed that no such respecification occurred and the 'normal' movements resulted from a good, but imperfect adaptation on the part of the animal to the handicap. It is therefore somewhat ironic that one of the more recent contretemps in the field, which also concerns the respecification of connections, though in embryos, not adults, has in its turn recently been resolved by close attention to anatomy. The argument, recapitulated by J. Feldman (London), was over Jacobson's elegant experiments apparently showing respecification of the dorso-ventral and antero-posterior polarity of frogs' eyes after very early rotation of the eye cup¹. Feldman and others working in M. Gaze's laboratory consistently failed to replicate Jacobson's electrophysiological evidence for normal retinotectal maps in the adult animals, and the conflict was resolved only when an examination of the eye itself revealed that rotated eyes are usually replaced gradually by new eyes which regenerate from residual unrotated tissue^{2,3}. Gaze and his colleagues examined their animals early, before

regeneration was complete; Jacobson examined his later: hence the conflicting results. In fact, the balance of the evidence now suggests that the polarity of neural tissue is irreversibly determined very early in development and rotation of embryonic tissue is one of the few manipulations that does not result in respecification: instead, the nerves tend to re-rotate *en route* to their targets.

The focus of controversy has now switched to the question of how far general properties such as polarity can account for the precision of individual connections between one array of nerve cells and another. There are two extreme positions on this. One is that given a fixed polarity and a chemical signal that determines the general direction of growth, the orderliness of connections can be ensured simply by the strict preservation of neighbour relations, possibly by general adhesiveness between growing fibres. The other is that nerve fibres are guided to their destinations by specific chemoaffinity between an individual innervating neurone and its target. Neither of these mechanisms however can account for the flexibility shown by growing nerve fibres in response not only to experimental manipulation but to the demands of normal growth.

For example, both the retina and the visual tectum of the frog *Xenopus* grow continuously throughout life. But the retina grows concentrically, whereas the tectum grows from rostro-lateral to caudo-medial; so in order to maintain an orderly map, connections between the two must be continuously broken and re-established (M. Keating, NIMR London). This rules out strict chemoaffinity and implies that incoming nerve fibres are ordered relatively rather than absolutely. That the ordering is relative with respect to the other nerve fibres as well as to the target follows from the results of removing either half of the retina, or half of the tectum in developing fish, amphibia or hamsters⁴. When the optic nerve fibres arrive at the tectum, they spread out over the whole tectum, or squeeze up into half of it, respectively, in an orderly fashion.

On the other hand, there is evidence against the preservation of neighbour relations by general adhesiveness. The ordering of nerve fibres is not always reflected in their physical relationships to one another: in both *Xenopus* and cat,

nerve fibres leave the retina in an orderly manner, become increasingly disordered on the way to the tectum (or in the case of the cat, the lateral geniculate), and re-establish order once more just before arrival^{5,6}. It is thus more as though fibres are assigned intrinsic positional values which govern their actual positions only in response to local cues, for example at the target. Furthermore, it is clear that the response may not result in the simple establishment of linear order. For example, in the optic chiasm of mammals, half the fibres of each eye follow a course to the contralateral side of the brain while the other half turn to make connections on the ipsilateral side. Similarly, even in the ribbon nerve of the cichlid fish, in which neighbour relations are rigidly maintained over most of the pathway, the fibres undergo systematic piecewise rearrangement at the point of their entry to the tectum (J. Scholes, Kings College London)⁷.

The one system in which neighbour relations do seem to be rigidly maintained throughout is the retino-tectal map of the chick. Not only does the retino-tectal projection remain orderly throughout its course, but, unlike those of hamsters and frogs, it fails to compensate for ablation: removal of part of the developing retina results in a corresponding area of denervated tectum (W.M. Cowan, Salk Institute)⁸. This inflexible behaviour on the part of the chick optic nerve has been the main support for the school of thought favouring a simple neighbour-relations mechanism for the specification of connections. The alternative point of view is that the chick retino-tectal projection is a less plastic manifestation of the same mechanisms that operate in fish, frogs and mammals.

This point of view is certainly easier to reconcile with other aspects of neural development in the chick itself. Nearest-neighbour guidance is plainly inadequate to explain the pattern of connections between spinal motor neurones and the muscles of the chick limb, which have been investigated in considerable detail in the laboratory of L. Landmesser (Yale University).

Like the eye, the spinal cord seems to

Miranda Robertson is associate editor of Nature.

*The Symposium of the British Society for Developmental Biology on Development in the Nervous System was held in Southampton on September 9-11 1980.

acquire an irreversible polarity very early in embryogenesis. If small sections of chick spinal cord are reversed before the outgrowth of motor nerve fibres, the axons will correct for the reversal and adjust their paths so as to connect with the muscles appropriate to their original position⁹. This is at first sight inconsistent with the results of Stirling and Summerbell¹⁰, who reversed the limb bud instead of the spinal cord and found that ingrowing motor neurones failed to correct for the reversal. Landmesser and her colleagues have suggested two ways in which this failure could be explained without abandoning the principle of early determination of polarity. The first is to postulate that because there is no conflict between the polarity of the nerve fibres and that of their environment until they actually enter the limb, nerve fibres growing into a reversed limb bud are simply not alerted to the reversal until it is too late for them to change their paths. Landmesser finds that nerve fibres from reversed segments of cord begin to adjust their courses soon after they have left the spinal cord, which suggests that the fibres are guided at least partly by morphogenetic signals that operate before they enter the limb. The second explanation for the conflict between Landmesser and Stirling is that there are limits to the extent of the reversal a nerve fibre can correct. Landmesser finds that after cord reversal limited to 2 or 3 segments, motor nerves can always correct their paths, whereas reversals involving 4–6 segments may defeat them. Her detailed investigations on the innervation pattern resulting from reversal of 4–6 lumbosacral segments suggest that the nerve plexus at the base of the limb may be crucial in sorting out the branching pattern of the ingrowing nerves, and that nerves that fail to enter the correct bundle at the plexus are usually the ones that innervate the wrong muscles. Even then, however, some nerves reach their own muscles by taking novel pathways in the limb.

These results plainly imply relatively specific local signals; but a recent ingenious attempt by Lewis to test for the presence of specific chemoaffinities in the chick wing raises the possibility of other interpretations. Lewis traced the fate of the brachialis longus inferior nerve in the wings of chicks in which one wing bud had been grafted onto the end of another before the outgrowth of motor axons¹¹. In a normal wing, this nerve branches into three at the elbow, the median nerve proceeding to the wing tip. In the experimental wings, however, the median nerve on arriving at the wing tip is confronted with a second elbow. But instead of growing onto the second wing tip, as would be predicted on the basis of chemoaffinity, the nerve branches again exactly as the brachialis branched at the first elbow.

There are three ways of explaining this result. Perhaps the growing median nerve contains components of the other two

branches which would normally be eliminated once the nerve reached its target, but which given a second chance home to their own muscles. This could be tested by tracing their central connections. The second possibility is that purely mechanical factors determine the branching, and chemoaffinity operates only very close to the target. The third possibility is that the median nerve is behaving like the optic nerve of an amphibian half of whose retina has been removed, and spreading out according to its relative positional values to innervate the entire limb. More detailed investigations will be needed to resolve these ambiguities.

Neurochemical differentiation

By contrast with the resistance shown by nerves to respecification after tissue rotation, developing neurones show a surprising degree of flexibility in the face of simple transfer up or down the antero-posterior axis. Most strikingly, Le Douarin and her colleagues (CNRS, Nogent-Sur-Marne) have shown that autonomic neurones from differentiated adrenergic ganglia of chick embryos can be induced by back-transplantation into an earlier host at a different position to express cholinergic functions, with appropriate changes in connectivity. One possibility that has excited a great deal of interest is that the switch in neurotransmitter specificity may be the consequence of the change in connectivity: that is, that it is induced by the target organ. An important source of support for this idea has been the work of Patterson and his colleagues on the neurochemical differentiation of rat sympathetic ganglion cell *in vitro*.

They have shown that in the absence of other tissues, the dissociated neurones develop adrenergic properties, but cholinergic differentiation can be induced by co-culture with certain non-neuronal tissues, such as heart, or by soluble factors released by them. The main argument against an induced switch in neurotransmitter synthesis rests on the alternative possibility that environmental factors, or target organs, are selectively enhancing the survival of cholinergic neurones from a heterogeneous population. (This argument will be discussed in greater detail in a later report of a CIBA symposium on the development of the autonomic nervous system.) While selective cell death is without doubt an important mechanism in neural development, it cannot in fact account for the phenomena described by the Harvard group, which have now been followed at the single-cell level *in vitro*. The real question is whether they play any part in normal embryonic development. Thus one of the most important advances reported at the Symposium was the morphological investigation of sweat glands in the rat foot pad, which show evidence of a change from adrenergic to cholinergic innervation between 7 and 14

days after birth (S. Landis, Harvard University). At the 7th postnatal day, the neural network surrounding the developing glands stains brightly with fluorescent markers for endogenous catecholamines; thereafter, catecholamine fluorescence wanes and acetylcholinesterase staining increases. These changes are paralleled by corresponding changes in the appearance of the vesicles in the nerve endings: predominantly small granular adrenergic vesicles give way to larger, clear cholinergic vesicles.

It is possible to quibble about these results: the interpretation of vesicle morphology is not easy, and in this case it is complicated by the fact that although the endogenous catecholamines disappear, a specific uptake system for exogenous ones remains. However the observations are at the very least consistent with a transition from adrenergic to cholinergic function during normal development *in vivo*, even if it has not been possible yet strictly to rule out the retraction of early adrenergic fibres and their replacement by new cholinergic ones.

Differentiation of glia

If experiments *in vivo* are essential for establishing the relevance of phenomena *in vitro*, experiments *in vitro* are equally important for dissecting the bewilderingly complex interactions that take place *in vivo*. An important prerequisite for such dissection is a battery of specific markers for distinguishing between interacting cell types; and progress in the development of cell-type specific antibodies has recently been responsible for some interesting advances in the study of neurone-glia interactions. M. Raff and his colleagues (University College London) have been able to demonstrate that astrocytes, ciliated ependymal cells and oligodendrocytes differentiate in dissociated cell cultures of 10-day-old embryonic rat brain with exactly the same timing as they do *in vivo*, showing that the events that lead to their differentiation are already determined by 10 days' gestation and are not disturbed by the subsequent disruption of their positional relationship.

J. Brockes (California Institute of Technology), investigating later stages in the differentiation of peripheral glia, has established the existence of two inducible biosynthetic systems in Schwann cells. Contact with large axons (that is, axons which they would normally myelinate) induces an increase by at least 1,000 fold in the synthesis of the principal myelin protein P₀. More surprisingly, purified Schwann cells can be induced by co-culture with primary muscle cells to synthesize acetylcholine. The observation that Schwann cells can synthesize acetylcholine is not new: after denervation of vertebrate muscle *in vivo*, they have been shown to migrate into the region of the endplate where they secrete acetylcholine¹³. The new contribution of Brockes's experiment is to

Cretaceous ammonites

from M. K. Howarth

AMMONITES are an extinct group of Cephalopods that flourished during the Mesozoic era 240 to 65 million years ago. Their world-wide distribution and speed of evolution, reflected in distinctive shell features, makes them the most useful macrofossils in the Mesozoic for relative dating of rocks. Rock divisions representing time intervals as small as about one-third of a million years can be recognized.

Ammonites from coastal exposures of Upper Cretaceous rocks in Zululand and Pondoland in South Africa are splendidly illustrated in a recent work by Klinger & Kennedy*. Abundant representatives of

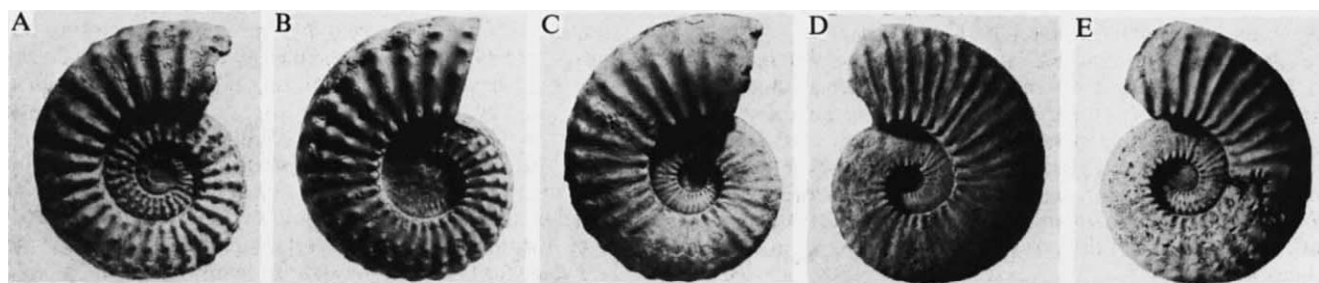
M.K. Howarth is in the Department of Palaeontology, British Museum (Natural History), London.

the subfamily Texanitinae were found in two areas 350 miles apart. Careful stratigraphical collecting showed that in each case there are complete series of transitions between the same four successive species, two of the ancestral *Texanites*, two of the successor *Submortonicerases*. Although the four species showed very high variability in both areas, there are small consistent differences between the same species in the two areas that are held to be of subspecific rank. It seems that the two populations were sufficiently isolated geographically to maintain these subspecific differences, but contact between them was enough to ensure that they evolved in parallel in specific and generic characters.

In the past ammonite systematics became unnecessarily complicated as generic and specific divisions were multiplied without any agreed method of control. This description of South African Upper Cretaceous ammonites is a good example of the new methods that have been applied during the past 25 years, involving accurate stratigraphical collecting and the use of single bed assemblages as a control to the species classification. It has been found repeatedly that the considerable morphological variation that has to be admitted to ammonite species, embraces many species and even genera of past ammonite systematists.

*H.C. Klinger and W.J. Kennedy *Annals of the South African Museum* 80, 1980.

A — *Texanites presoutoni* sp. nov.; B — *Texanites soutoni* (Bailey); C — *Submortonicerases woodsii* (Spath); D — *Submortonicerases condamyi* (Collignon); showing progressive evolution from A, the oldest, to D, the youngest. E — *Submortonicerases woodsii* (Spath).



show that it is almost certainly the muscle that induces acetylcholine synthesis in the Schwann cell. The identity of the inducing factor(s) is, here, as elsewhere, unknown.

It is an exasperating fact that while the chemical mediators of induction in embryos remain unknown, most of the known and characterized growth factors discovered through assays *in vitro* have no well established role *in vivo*. The most recently discovered of these factors is a component of the brain and pituitary that causes proliferation of Schwann cells and astrocytes *in vitro*¹⁴. Brookes has now raised a monoclonal antibody against it, which will make it possible to purify and sequence it; and to discover what, if any, its function is *in vivo*.

Solar oscillations

from H.B. van der Raay

KNOWLEDGE of the Sun, our closest star, is based on observations of its surface properties. The temperature, magnetic field and constituents of the outer layers can be deduced by spectroscopy and, combining these data with estimates of size and mass, a model of the Sun's properties can be formulated. The model fits not only the observed surface properties but also predicts interior characteristics such as density and temperature. From these parameters and rates for the nuclear fusion reaction which converts hydrogen to helium and provides the energy output of the Sun the number of solar neutrinos associated with the nuclear burning may be readily found. An experimental verification of the standard solar model thus became possible. Unfortunately, as is well known, the experiment designed to detect these solar neutrinos failed to record the numbers predicted.

An alternative way to probe the solar interior is by the detection of solar oscillations in a manner analogous to terrestrial seismology. The pressure waves associated with the oscillations, especially if of long period, penetrate deeply into the

solar interior and convey information about the internal structure. Here again observations and predictions from the standard model appear contradictory. Does this imply that the theory is incorrect or is it the interpretation of the observations that is at fault?

Long period oscillations of the sun were discovered by Birmingham¹ and Crimean² groups, working independently, in 1976. Both groups measured the line of sight Doppler shifts of certain Fraunhofer absorption lines and, since observations were made on the integral solar disk, concluded that these observations corresponded to simple radial oscillations of the sun. The measured period of 160 minutes raised an immediate conflict with the standard solar model since if these were simple radial oscillations the longest period predictable was approximately 60 minutes. The Cambridge theorists³, however, pointed out that the observations could be interpreted in terms of more complex g mode oscillations.

Since these early observations, studies of line of sight velocities of the solar surface have continued. The Crimean group have found evidence that the 160 minute oscillation has persisted to the present day

1. Jacobson, M. *Science* 155, 1106 (1967).
2. Gaze, R.M., Feldman, J.D., Cooke, J. & Chung, S.-H. *J. Embryol. exp. Morph.* 53, 39 (1979).
3. For brief summary see Robertson, M. *Nature* 278, 778 (1979).
4. *Neurosci. Res. Prog. Bull.* 17, (1979).
5. Fawcett, J.W. *J. Physiol.* 306, 32P (1980).
6. Horton, J.C., Greenwood, M.M. & Hubel, D.H. *Nature* 282, 720 (1979).
7. Scholes, J.H. *Nature* 278, 620 (1979).
8. Cowan, W.M., Rogers, L.A. & Kelly, P.J. *J. comp. Neurol.* 155, 127 (1974).
9. Lance-Jones, C. & Landmesser, L. *J. Physiol.* in the press.
10. Stirling, R.V. & Summerbell, D. *Nature* 282, 640 (1979).
11. Lewis, J. *Zoon* 6, 175 (1980).
12. Le Douarin, N.M. *Nature* 286, 663 (1980).
13. Miledi R. & Slater, C.R. *Proc. R. Soc. Lond. B* 169, 289 (1968).
14. Brookes, J.P., Lemke, G.E. & Balzer, D.R. jun. *J. biol. Chem.* 255, 8374 (1980).

H.B. van der Raay is in the Department of Physics, University of Birmingham UK.

with the same phase, although with a substantially decreased amplitude. This is confirmed by a group working at Stanford⁴. The Birmingham group have, however, failed to confirm this result and find many long period oscillations which vary in period, phase and amplitude from one day to the next⁵.

All these observations necessarily suffer from the fact that the sun is only visible for part of the day and, as a consequence, the data sets are modulated by a 24 hour period. Any periods found which are harmonics of a day thus need to be considered most critically. 160 minutes is exactly 1/9 of a day — could this oscillation appear simply as the result of the method of analysis? The Crimean and Stanford groups, aware of this possibility, point out that the period is now found to be 160.01 minutes.

At the other end of the time scale are the five minute oscillations first discovered by Leighton in 1960. A detailed study of this region over some three years by the Birmingham group⁶, showed that these five-minute (~ 3 mHz) oscillations had a well defined structure and consisted of >20 well defined discrete frequencies with a mean spacing of alternate lines of 135.6 μ Hz. Interpreting these data in terms of the then current solar models Iben and Mahaffy⁷ and Christensen-Dalsgaard *et al.*⁸ suggested a heavy element abundance $z \approx 0.004$ in contrast to the standard model value of $z = 0.02$.

The solution to the 24 hour modulation problem may be found in three ways: (a) by placing many observation stations around the globe, (b) by observing at one of the earth's poles, (c) by operating from a suitable space satellite. Each of these solutions have both advantages and disadvantages: (a) would require redundancy at any given longitude to overcome weather problems, (b) is subject to atmospheric effects as the sun is always close to the horizon, (c) includes all the technical problems associated with a space program. Also the costs involved increase rapidly as one proceeds from (a) to (c).

The polar solution has been taken by Grec *et al.* and is described in this issue of *Nature* (see p.541). The stresses of an austral summer were amply rewarded by the results of an excellent experiment which resulted in 120 hours of uninterrupted

observations. The preliminary analysis confirms the detailed structure and spacing found in the five-minute oscillation. However, those data relating to the longer periods do not show convincing evidence for the existence of a 160 minute oscillation. It is only when a superposed epoch analysis similar to that used by the Crimean and Stanford groups is used that the 160 minute period emerges. Admittedly the 24 hour modulation associated with daytime observation is no longer present in these data, but the earth does still rotate about its axis once every 24 hours, even at the pole. Hence any instrumental or atmospheric effects would have to be clearly eliminated.

Interpretation of the five-minute structure depends on the assignment of the modes observed. All the Doppler shift measurements are strongly biased to low order modes since the integral disk is observed. Hence it is customary to assume $l_0, l_1, \dots$ modes, where l_0 is the simple radial oscillation l_1 alternate radial and axial expansions and contractions and higher l values correspond to more complex shape deformations. Once l has been assumed then for a given frequency ν , the number of radial overtones, n , may be estimated and the results compared with theory.

In a revival of the standard solar model Christensen-Dalsgaard and Gough present a paper in the present issue of *Nature* (see p.544), in which the standard value for the

heavy element abundance is retained, $z = 0.02$, and the observational structure of the five-minute oscillations is fitted by taking into account the effect of the solar atmosphere. However it is clear from the models listed that these are very insensitive to the observed frequency separation. Indeed it appears that the only significant change in $\Delta\nu$, the frequency separation, occurs when low z values are considered. At the recent Eslab symposium⁹ a mean spacing of $135.2 \pm 0.2 \mu$ Hz was reported which would still appear to favour the $z = 0.004$ value. A direct comparison of the predicted frequencies with the 25 which have now been measured to an accuracy of 1:10³ may yield a more conclusive test as to which model to use.

All interpretation of the data depends critically on the correct identification of the mode concerned. This may be experimentally determined by considering two dimensional observations of the solar disk. Such work is at present in progress¹⁰ and will hopefully resolve any mode identification problems. The experimental observations of independent groups have firmly established the discrete structure of the five-minute oscillations although the mode identification and reconciliation with theory is still unsatisfactory.

Clearly if we do not understand our own closest star, the implications on the whole field of cosmology are enormous, and continued efforts by both theorists and experimentalists are urgently required.

Coronavirus come of age

from B.W.J. Mahy

ALMOST thirty years after the pioneering work of A.W. Gledhill and C.H. Andrewes at the Mill Hill laboratories established the essential features of murine hepatitis virus (MHV) infection (*Brit. J. exp. Pathol.* **32**, 559) the first international conference on the coronavirus group, of which MHV is the best studied member, has been held in Germany*. The meeting consolidated much new and interesting data on a group of viruses responsible for a wide range of acute and chronic diseases. These presently include respiratory and enteric disease in humans, bronchitis in birds, transmissible gastroenteritis and encephalitis in pigs, diarrhoea in calves and dogs, peritonitis in cats, and both demyelinating encephalitis and hepatitis in rodents. A recent report of the isolation of coronaviruses from the brains of multiple sclerosis patients (Burks *et al. Science* **209**, 933; 1980) has intensified interest in the group.

During the first half of the meeting, on

structure and replication, it became clear that, in addition to the characteristic morphological features of the group, coronaviruses from whatever species are unified by the possession of a large infectious single-stranded genome of 6 to 7 $\times 10^6$ molecular weight. Both the mechanism of expression of this genome, and the protein products, have unique features. M.C. Lai (Los Angeles) for a murine coronavirus and S.I.T. Kennedy (La Jolla) working with avian infectious bronchitis, each presented evidence that the genome is positive-stranded, with a capped (m⁷G) 5'-terminus and a stretch of poly(A) at the 3'-terminus. In infected cells six virus-specific RNA species are consistently found, ranging in molecular weight from 0.6 $\times 10^6$ up to genome size. T₁ ribonuclease mapping studies (S.I.T. Kennedy and J.L. Leibowitz, La Jolla) show that these RNAs form a 'nested set', the sequence of each RNA being contained

1. Brookes, J.R., Isaak, G.R. & van der Raay, H.B. *Nature* **259**, 92 (1976).
2. Severny, A.B., Kotov, V.A. & Tsap, T.T. *Nature* **259**, 87 (1976).
3. Christensen-Dalsgaard, J. & Gough, D.O. *Nature* **259**, 89 (1976).
4. Scherrer, P.M., Wilcox, J.J., Kotov, V.A., Severny, A.B. & Tsap, T.T. *Nature* **277**, 635 (1979).
5. Brookes, J.R., Isaak, G.R. & van der Raay, H.B. *Mon Not R. astr. Soc.* **185**, 1 (1978).
6. Claverie, A., Isaak, G.R., McLeod, C.P., van der Raay, H.B. & Roca Cortes, T. *Nature* **282**, 591 (1979).
7. Iben, I. & Mahaffy, J. *Astrophys. J.* **209**, 39 (1976).
8. Christensen-Dalsgaard, J., Gough, D.O. & Morgan, J.G. *Astr. Astrophys.* **73**, 121 (1979).
9. Claverie, A., Isaak, G.R., McLeod, C.P., van der Raay, H.B. & Roca Cortes, T. *XIV Eslab Symposium* (1980).
10. Brookes, J.R., Isaak, G.R. & van der Raay, H.B. *XIV Eslab Symposium* (1980).

B.W.J. Mahy is Huddersfield Lecturer in Special Pathology at the University of Cambridge.

*An international symposium on the 'Biochemistry and Biology of Coronaviruses' sponsored by the Deutsche Forschungsgemeinschaft was held in the Institut für Virologie und Immunbiologie, University of Würzburg from October 15th to 18th, 1980.

within the sequences of all larger RNAs; the 3'-termini of all the RNAs are common, and they all extend inward from the 3' end of the genome. *In vitro* translation experiments by S. Siddell (Würzburg) and B. van der Zeijst (Utrecht) showed that each subgenomic RNA is a messenger species which specifies a single primary protein product translated from its unique 5'-terminal sequence. Nothing is known at present as to the mechanism by which these subgenomic RNAs are synthesised, although UV target size measurements suggest that each is independently initiated on a negative strand template, and not derived by processing from a larger species (van der Zeijst). A membrane-bound RNA-dependent RNA polymerase activity present in cells infected with a porcine coronavirus was described by D. Brian (Knoxville), but as yet no evidence as to the nature of replicative intermediate molecules has been obtained.

Of the three well-defined virion proteins, one is a 60K nucleocapsid protein phosphorylated by a virion-bound protein kinase, and the others are envelope glycoproteins. The largest (90K) forms the petal-like structures (peplomers) of the 'crown' (from which the group derives its names), and apparently has a conventional mode of synthesis similar to the glycoproteins of other enveloped viruses. The smaller glycoprotein (23K) is embedded in the envelope lipid bilayer, and is largely unaltered by treatment of the virus particle with bromelain, which removes the peplomers; this protein thus fulfils a similar role to that of the membrane protein of other enveloped viruses such as influenza. Glycosylation of this coronavirus membrane protein has unique features. R. Rott (Giessen) showed for a bovine coronavirus that the 23K protein contains no fucose or mannose. H. Niemann and H. Klenk (Giessen), P. Rottier (Utrecht) and K. Holmes (Bethesda) all reported that synthesis of the 23K glycoprotein is not inhibited by tunicamycin; this indicates that, in contrast to all other virus glycoproteins studied so far (including the 90K corona-virus protein) dolichol-linked N-acetylglucosamine plays no part in biosynthesis. L. Sturman (Albany) showed that in the presence of tunicamycin virus particles still matured, but were released from the cells as 'spikeless' virions lacking the 90K protein.

Comparisons between the genomic and subgenomic RNAs of coronaviruses differing in pathogenic potential or species of origin were reported by several workers. T₁ ribonuclease mapping studies by B. Lomniczi (Budapest) gave rise to at least 11 different genome RNA fingerprints within 13 isolates of avian coronavirus originally classified as infectious bronchitis by pathological criteria. However it is likely that this technique provides too fine an analysis of genome structure for useful strain comparisons. S. Weiss (Philadelphia) described the *in vitro*

Molecular lines in the red

from a correspondent

THE FIRST OBSERVATIONS of molecular emission in the far infra-red (IR) from an interstellar cloud have been made by a team at the University of California (D.W. Watson, J.W.V. Storey, C.H. Townes, E.E. Haller and W.L. Hansen *Astrophys. J.* **239**, L129; 1980). These observations were made from the NASA Kuiper Airborne Observatory in January 1980 at an altitude of 12.5 km, using a liquid helium-cooled Ge:Sb photodetector in a metal-mesh, scanning Fabry-Pérot interferometer. The two $J = 21 \rightarrow 20$ and $J = 22 \rightarrow 21$ rotational lines of CO were observed in emission at 124 and 119 μm from the Kleinmann-Low region of the Orion molecular cloud. The CO molecule in interstellar clouds has previously been observed in the millimetre and submillimetre regions of the spectrum, where it has been shown to be one of the principal molecular species containing carbon in dense molecular clouds such as Orion. The significance of the new 124- and 119- μm observations is that for these rotational transitions to be observable at all, the CO must be much

hotter (500–1,000K) than the temperature of the CO molecules observed in the microwave ($\sim 100\text{K}$). These high temperatures indicate that the CO observed in the far IR is present in a post-shock gas, corroborating earlier evidence obtained from H₂ observations that a shock wave is propagating into the Orion cloud.

This observational technology opens up an entire new spectral range for observations of molecular species in the interstellar medium. Until now observations of interstellar clouds have been limited mainly to the microwave and radio regions at the long-wavelength end of the spectrum, and to the visible and near ultra-violet regions at the short end of the spectrum. The IR region of the spectrum is a rich arena for remote-sensing applications, but it has been essentially closed to interstellar observations by a combination of detector problems and atmospheric absorption. The development of far-IR techniques for observation of the interstellar medium increases the potential for new discoveries concerning the structure, dynamics, chemistry and evolution of interstellar clouds. □

synthesis of a representative DNA copy of the genome of MHV strain A59. Using this cDNA probe, she found considerable sequence homology with other murine coronaviruses and a low level of homology with 229E virus, a human coronavirus. Extension of such studies to the virus isolates from multiple sclerosis brain tissue, which were obtained by passage of the tissue in mice, should help to identify the origin of these agents.

The second half of the meeting was devoted to persistency and pathogenesis. Although little is known of the molecular mechanisms underlying organotropism and pathogenesis following coronavirus infection, some advances were reported. Coronavirus-induced disease is very dependent upon the age at infection, genetic background, and route of inoculation of the host. K. Pickel (Würzburg) showed that mice were fully susceptible to fatal JHM virus infection up to the age of 21 days, but thereafter became fully resistant. Resistance could be induced by transfer of immune spleen cells from adult mice to baby mice, or by priming of adult non-immune spleen cells in baby mice with UV-inactivated JHM virus, but not by non-immune spleen cells alone. R. Knobler (La Jolla) confirmed that fatal encephalomyelitis induced by JHM virus is an autosomal dominant trait; the ability of the virus to induce demyelinating lesions

depended upon the cell tropism of selected mutants to replicate in oligodendroglial cells. H. Wege (Würzburg) reported that injection of JHM *ts* mutants into preimmunised rats by the intracerebral route causes a high rate of subacute to chronic demyelinating disease with similarities to virus-induced demyelination in man. Rats survive normally after intraperitoneal injection of similar virus doses.

Several investigators have studied the mechanism of resistance at the cellular level. F. Bang (Baltimore), who had shown as early as 1960 that peritoneal macrophages taken from MHV resistant mice were also resistant to infection *in vitro*, now reported that macrophage resistance is also dependent upon associated lymphocyte action and may also involve interferon. Extension of these studies from macrophages to primary mouse hepatocyte monolayer cultures by H. Arnheiter (Zürich) confirmed that the hepatocytes were genetically resistant to virus infection as were the mice themselves. Although interferon treatment reduced virus titres in susceptible cell cultures, addition of anti-interferon antiserum did not augment the susceptibility of resistant hepatocyte cultures. Thus it can be concluded that such cells are intrinsically resistant to virus infection, though by what mechanism remains to be determined.

In contrast to other positive strand RNA viruses, the corona-viruses readily establish persistent infections in the host. For example, chronic hepatitis or chronic demyelination of the central nervous system occur with certain virus-host combinations. Not surprisingly, persistent infections are relatively easy to establish in cell culture *in vitro*, and such model systems have some unusual features. K. Holmes (Bethesda), N. Hirano (Morioka) and S. Stohman (Los Angeles) each reported that in cloned cell cultures persistently infected with MHV only 10–20 percent of the cells express coronavirus antigens detectable by immunofluorescence, but 100 percent are resistant to superinfection with homologous virus. G. Chalon-Larsson (Ottawa) has obtained very similar results with a human

coronavirus (229E) persistent infection of L 132 cells. Further characterisation of such *in vitro* systems is sure to provide much-needed insight into the way these viruses establish such an intimate relationship with the host. Summing up the meeting, D. Tyrrell (Harrow) emphasised the very wide range of diseases now known to be caused by these viruses, and the value of their study as models of virus pathogenesis. As the meeting convener, V. ter Meulen (Würzburg) pointed out, virtually every laboratory currently working with coronaviruses was represented amongst the seventy participants, and the collective presentation of their data undoubtedly served to strengthen and unify their purpose in unraveling the molecular biology of this unique group of viruses.

transposition is reasonably low, it should be possible to build a *Drosophila* strain that lacks copia sequences. Young has made a start to this interesting, but very tedious experiment: at the time of the meeting a strain with only six copia sequences had been made and the flies were apparently no worse for their experience. Only time, and hard work, will tell whether or not copia sequences are necessary for some aspect of *Drosophila*'s existence.

The second genetic approach stems from the exciting discovery that at least one well known mutation (white-apricot) is associated with an insertion of copia into the white gene. B. Judd and P. Bingham (Research Triangle) have mapped this copia element within white to the same region as maps the *w^{ad}* allele and W. Gehring's laboratory (Basel) has found that revertants of *w^{ad}* may be accompanied by loss of the copia element from this site.

The role of the insertion of nomadic middle repeat sequences into genes as a cause of spontaneous mutation in *Drosophila* was also highlighted by the molecular study of the bithorax region. W. Bender (Caltech) has now cloned over 145 kilobase pairs of this region and has used these clones to analyse the nature of some of the many mutant bithorax genes. Two (*bxd¹* and *bxd⁵*), both spontaneous in origin, result from large insertions (of about 10 kb) into the gene. Although the insertion sites are not identical they are reasonably close together and each insertion is a repetitive DNA sequence. A spontaneous reversion of *bxd* turns out to have lost most, though not all, of its insertion. It must be most gratifying to E. B. Lewis, who is responsible for the very detailed genetic analysis of this complex locus, to see how well the molecular analysis of these mutations is confirming the genetic data. For example, the dominant mutation *Cbx* was recovered, after X-ray mutagenesis, together with a recessive *pbx* allele: Bender has now shown that *pbx* is a deletion and that the sequence removed from the *pbx* site has been inserted some kilobase pairs away in reverse orientation to give the *Cbx* mutation. Reversion of *Cbx* has been accompanied by an inversion broken within this inserted sequence.

It is to be hoped that the molecular analysis of long regions of the genome will throw some light on the old question of the relationship between genetic organisation and chromosome structure. P. Spierrer (Geneva) recounted his latest travels along the chromosome in the region of the genes coding for xanthine dehydrogenase (*rosy*) and acetyl cholinesterase. Over 300 kb of DNA (three 'Benders') have been covered and this spans eight polytene chromosome bands and as many characterised genes. As dramatically as any other this experiment demonstrates the variation in location of middle repeat DNA sequences. The DNA cloned for this walk came from two different 'wild type' stocks of *D.*

Drosophila at Kolymbari

from M. Ashburner

FOR the second time the Greek Orthodox Academy at Kolymbari, on the northern shores of Crete, was host to a meeting of *Drosophila* biologists*. Not surprisingly, the scientific sessions were dominated by discussions of the molecular structure of the *Drosophila* genome. Restriction maps and nucleotide sequences were so common that I am sure more than one participant considered that his colleagues, but naturally not himself, had fallen into the trap so carefully avoided by Dr Watson, of confusing hard work with hard thinking. Nevertheless some very interesting facts were brought to our attention: interesting if only for the reason that they were quite unexpected.

There can be little doubt that the major challenge to conventional genetic wisdom revealed by the molecular analysis of the genome of *Drosophila*, and other eukaryotes, has been the discovery of repeated DNA sequences of no fixed abode. The 'type' of these sequences, as far as *Drosophila* is concerned, is that dubbed 'copia', a sequence of some 5 kilobase pairs which is present, in the typical strain of *D. melanogaster*, roughly 30 times. Comparison of the chromosomal locations of this sequence in different strains of *D. melanogaster* has shown that these differ between, and even within, strains.

Studies on the sequence organisation of copia, and similar elements, by G. Rubin's laboratory (Harvard Medical School) have revealed striking similarities between copia, a dispersed middle repeat sequence of yeast known as Tyl, and integrated vertebrate retroviruses. Copia is flanked by

a direct terminal repeat of 276 base pairs and a comparison of the nucleotide sequence of sites into which copia has inserted reveal that insertion is accompanied by a direct duplication of five base pairs of the 'target' sequence. Other nomadic middle repeat sequences of *Drosophila* appear to have a similar overall organisation to copia but to generate duplications of the insertion site of different sizes (for example four or seven base pairs). The similarities between these elements and bacterial IS sequences are striking; whether these similarities reflect the mechanisms IS sequences and the nomadic eukaryotic elements use for transposition is not yet known.

Copia, and some other nomadic middle repeat sequences of *Drosophila*, were originally cloned by virtue of the fact that their RNA transcripts are particularly abundant, at least in tissue culture cells. Work in both M. Young's laboratory (Rockefeller University) and in Rubin's has now shown that several different transcripts complementary to copia can be found in cells: whether these can all come from any single copia sequence is not known. For many years the function of RNAs complementary to copia has been the subject of some considerable discussion. They are clearly not translated in proportion to their mass yet, as work by both Rubin and J. Lengyel (University of California, Los Angeles) has shown, these RNAs can be translated into polypeptides in a cell free system.

Two genetic approaches to understanding the function of these nomadic sequences were discussed at Kolymbari. Since different stocks of *D. melanogaster* differ in the location of their copia sequences, and since the frequency of copia

M. Ashburner is in the Department of Genetics, University of Cambridge.

melanogaster; Oregon-R and Canton-S. Overall seven different insertions of middle repeat DNA were found but none were in the same location in both stocks: indeed many were polymorphic within a stock. Yet for non-repetitive DNA these two stocks show only a fraction of a percent divergence in sequence.

A rather different type of transposable element in *Drosophila* was described by G. Ising (Lund). This element, called TE1, is huge, it carries at least two genes (white and roughest) normally adjacent in the X. It arose spontaneously some years ago as a transposition of w^a and rst^+ from the X chromosome to chromosome two and, since then, has been merrily hopping around the genome with a frequency of about $1:10^4$. Ising has mapped over 150 different sites into which it has inserted, insertion often being accompanied by mutation of the 'target' gene. Just how TE1 moves, or how it can be lost altogether from the genome, is rather mysterious but there is hope that the cloning of one end of TE1 fortuitously inserted very near one of the heat shock genes (M. Goldberg, Basel) will help to solve this mystery.

As at Kolymbari two years ago the analysis of genes of known function, known at least in terms of their protein products, continues to give great pleasure to participants and onlookers alike. Those few genes in *Drosophila* whose activity is so dramatically induced by heat shock are by far the best known. Cloning has confirmed earlier genetic conclusions that those coding for the four small heat shock proteins are very closely linked. R. Morimoto (Harvard) described that their coding sequences are all contained within a 13 kb fragment. Detailed comparison of

the DNA sequences upstream of the 5' ends of these genes with those of genes coding for three other heat shock proteins is revealing homologies some 125 bases upstream from the 'TATA box' sequence.

The clones of the 'major' heat shock gene (Hsp70), a gene that is repeated at each of two different loci in *D. melanogaster*, are now beginning to be used for a number of quite different studies, for example of gene evolution (D. Ish-Horowicz, ICRF, London), nucleosome spacing (M. Noll, Basel) and chromatin organisation revealed in the pattern of sensitivity to nucleases (S. Elgin and C. Wu, Harvard). Yet early hopes that a comparison of the sequences adjacent to different Hsp70 copies would quickly reveal those sequences important for the control of transcription appear to have been too optimistic, since variant Hsp70 genes have been isolated that can be shown to be functional, yet lack a characteristic class of sequences normally found 5' to the coding region (M. E. Mirault, Geneva). On the other hand the conference heard from J. Liss (Cornell) that the insertion of a small (406 base pair) sequence, which includes the first 63 base pairs of the translated Hsp70 gene and material 5' to it, into a particular repetitive DNA sequence found adjacent to some Hsp70 genes results in the induction, by heat shock, of transcription of the middle repeat sequence.

Unfortunately, the function of the heat shock proteins remains unknown. Their importance for the cell in the recovery of normal protein synthesis after the heat shock was shown by S. Lindquist (Chicago). The incorporation of certain amino acid analogues into the heat shock

proteins apparently blocks their post-transcriptional processing and normal accumulation in the nucleus; it also blocks the recovery of normal protein synthesis after the heat shock. Lindquist suggests that normally these proteins may be nucleic acid binding proteins. Recently, rather similar responses to environmental shock have been found in organisms as different as yeast and man. A. Tissières (Geneva) discussed evidence from fingerprints of 'heat shock' proteins from different animals that indicates that at least Hsp70 may be conserved.

Progress is also slow in discovering the mechanisms of induction of heat shock protein synthesis. T. Kornberg (University of California, San Francisco) has characterised an *in vitro* system using isolated *Drosophila* tissue culture cell nuclei in which the *E. coli* RNA polymerase directed transcription of the heat shock genes is responsive to the addition of factors from heat shocked, but not control, cells. The nature of these factors (or factor) is yet to be elucidated.

Other genes that attracted attention at Kolymbari were those coding for the salivary gland 'glue' proteins and the proteins of the egg shell of *Drosophila*. Each had their surprises. A component of the salivary gland glue, known as SGS-4, is coded for by a gene on the X chromosome and is of interest for several reasons. G. Korge (Munich) has characterised mutations that normally fail to express this gene yet will do so when the mutant gene is made heterozygous with a wild type allele, at least under certain conditions. This is rather similar to the transvection affect described for the bithorax locus by E. B. Lewis 25 years ago. The coding region for SGS-4 has

100 years ago



Fanduri — a Dorey papuan



Epa — a Village of the Mahori papuans.

Pictures from "New Guinea: What I did and what I saw" by L. M. D'Alberty reported in *Nature* 23, 16 December, 1933, 1880.

been cloned in Stanford and is being used for the analysis of this entire region of the genome by S. Beckendorf (University of California, Berkeley) and for detailed studies of the gene itself by M. Muskavitch (Stanford). A surprising result is that the transcript of SGS-4 differs in size in different strains of *Drosophila*. This results, it would appear, from the fact that within the coding sequence there is a 21 base pair sequence whose repetition frequency can vary between different SGS-4 alleles. Although nothing is known of the chemistry of the protein this must reflect a sequence of seven amino acids repeated a varying number of times.

The mutation characterised by Korge, that fails to make SGS-4 protein when homozygous turns out to be a small deletion, but of a sequence over 200 base pairs away from the 5' end of the messenger RNA. The importance of this region is indicated by the fact that two different expressed alleles of SGS-4 differ by a single

base pair substitution in this region and by a factor of two in the abundance of their mRNAs.

The egg shell protein genes, under study by A. Spradling (Carnegie Institution, Washington) and in F. C. Kafatos' laboratory (Harvard) also surprised us for they are amplified in the tissue in which they are expressed. Spradling has found that the amount of DNA amplified extends at least 30 kb on each side of the genes themselves, but the degree of amplification falls off, from a peak of some 60 fold, in both directions away from the coding sequences. This, and the fact that the amplified DNA has an identical restriction pattern to non-amplified DNA, may indicate that the process is chromosomal, rather than extra-chromosomal as in oocyte ribosomal genes in many organisms. Yet the Harvard laboratory (G. Thiroes and R. Griffin-Shea) have found that amplification of another pair of egg shell protein genes is accompanied by their

premature transcription; but that these early transcripts are not translated and, indeed, decay as development gets under way. Only at the appropriate developmental stage several hours later does transcription resume to produce mRNAs that are subsequently translated.

What is the meaning of this? It implies some relationship between amplification and early transcription and also indicates the existence of a translational control at the early developmental stage.

Like all good meetings, at the end there were far more questions than answers. As is right and proper we were treated to many very preliminary data whose place in the jigsaw puzzle is yet undiscovered. The great success of the Kolymbari meeting was in bringing together molecular and classical *Drosophila* biologists to discuss their data from their different perspectives and we can only hope that some of today's mysteries will be clearer to us when we next meet.

Comets and the origin of life

from N.J. McNaughton and C.T. Pillinger

COMETS are the most spectacular bodies in our solar system and the anticipated return of Halley's Comet in 1985-6 has already proved a catalyst for increased cometary research. As the comet nucleus contains, perhaps with the exception of phosphorus, the necessary ingredients for life on this planet, the pundits of earthbound chemical evolution and cometary science found common ground at a recent colloquium* entitled 'Comets and the origin of life'.

Although almost all of our observations of comets have been obtained from or near the Earth, the accumulated data yields a basic understanding of the structure, chemistry and physics of the comet nucleus and tails. Perhaps the most significant point to emerge from new observations is the apparent constancy of comet's chemical composition.

A'Hearn (University of Maryland) observed that the rate of production of certain chemical species, particularly CN, C₂, C₃ and OH, from the comet nucleus near the sun is uniform irrespective of the type of comet and its gas to dust ratio. The spectroscopic continuums of comets of different ages are also strikingly similar (Donn, NASA-Goddard). It is not yet clear whether this uniformity is due to the nature of the chemical reactions in the coma or if it implies that comets are derived from a constant composition reservoir. How the latter interpretation fits with a recently announced theory for the origin of comets (Biermann *Royal Society Meeting on Planetary Exploration*, Munich, Nov. 4-5, 1980) is not clear. Biermann's new hypothesis predicts cometisimals as the

possible end product of a fragment of protosolar nebula collapsing in a relatively low magnetic flux. According to the same theory, planetary systems would be produced in a large magnetic flux and there seems to be no way for both planets and comets to be produced together.

The current interest in comets has promoted a shift in emphasis among the prebiotic synthesis investigators — low temperatures and photon or proton irradiation appear to be the order of the day. Reproducing cometary compositions and environments in the laboratory, the Maryland group and Greenberg (Leiden University, Netherlands) have synthesised 'life-like' organic molecules. Long chain hydrocarbons, nitrogenous compounds and carboxylic acids have been identified spectroscopically among the products of reactions carried out at temperatures as low as 15°K. Given that similar molecules could only be produced on the Earth under a strongly reducing protoatmosphere (which the Levine group from Virginia calculate would be short-lived due to the high reactivity and abundance of OH), the introduction of such molecules via comets into a less drastic protoatmosphere offers a real, and in some cases preferred, mechanism for the beginning of life on Earth. Speculations on this theme range from the controversial — Wickramsinghe (University of Cardiff) envisaging comets as the carriers of already living organisms — to efforts to calculate possible mass inputs (Lizcano-Aranjo, Houston).

Definitive evidence on the relationship between comets and the origin of life must

await the eventual laboratory analysis of cometary material. The identification of microscopic stratospheric and deep-sea particles of extraterrestrial origin by Brownlee (University of Washington) offers, however, the first real samples of probable cometary debris. The minute size and rarity of unaltered Brownlee particles preclude the use of most conventional analytical procedures, and substantial refinements to existing or even new techniques (such as the CD₄ carbon isotopic method pioneered at the University of Cambridge), are required before even the Brownlee particles can be fully exploited.

The alternative to analysing cometary material found in the terrestrial environment is *in situ* measurements from spacecraft. The planned NASA/ESA international mission to rendezvous with Halley's Comet which would have allowed analyses as sophisticated as those needed for origin of life studies will now not take place. ESA's fast flyby of Halley (the Giotto mission) will undoubtedly produce information on the composition of the gas and solid phases to fuel further laboratory based studies but is unlikely to provide definite answers to the question of whether comets were involved in the origin of life on Earth.

CORRIGENDUM

Edward Herbert would like to point out that he was not the sole author of *Biosynthesis and processing of cellular and viral polypeptides* (News and Views, 13 November, 288, 115, 1980) and would like to acknowledge the co-authorship of Gunther Kreil (Salzburg) and the help of Carolyn Carter and Eckard Wimmer (Stoneybrook, New York).

N.J. McNaughton and C.T. Pillinger are in the Department of Earth Sciences, University of Cambridge.

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ARTICLES

Solar oscillations: full disk observations from the geographic South Pole

Gerard Grec*, Eric Fossat[†] & Martin Pomerantz[‡]

* Département d'Astrophysique de l'IMSP, ERA 669 du CNRS, Université de Nice, Parc Valrose, F-06034 Nice Cedex, France

[†] Observatoire de Nice, BP 252, F-06007 Nice Cedex 2, France

[‡] Bartol Research Foundation of the Franklin Institute, University of Delaware, Newark, Delaware 19711

Observing conditions at the geographic South Pole enable modes of global solar oscillations and theoretical models of the internal solar structure to be identified.

THERE are only two methods available for probing the interior of the Sun to test the theoretical models of the internal solar structure. Such calculations have been based on externally observable parameters ($M_{\odot}$, $L_{\odot}$, $R_{\odot}$, $t_{\odot}$) plus some speculations regarding the relative abundances of certain elements, such as metals and helium¹.

The first technique requires the exceedingly difficult measurement of neutrinos emanating from thermonuclear reactions in the solar core. After much effort, Davis² established an upper limit of the solar neutrino flux (1.75 ± 0.4 SNU) that is somewhat below original expectations. These neutrinos convey information only about the central region of the Sun, where the time scale of temporal variations is enormous ($\sim 3 \times 10^7$ yr).

The second approach attempts to detect and identify normal modes of global solar oscillations arising from internal processes. These pulsations characterize phenomena in the layers in which dynamical processes can cause effects that produce visible manifestations on the solar surface³.

Efforts to detect global solar oscillations through observation of luminosity variations⁴⁻⁷ were unsuccessful because the amplitudes are far smaller than the transparency fluctuations of the Earth's atmosphere.

Another technique utilizes continuous determinations of the apparent solar diameter⁸⁻¹². These experiments have produced positive results which have been attributed to underestimates of the effects of atmospheric fluctuations¹³⁻¹⁶.

Finally, various Doppler shift measurements of large-scale velocity fields have been carried out either with spatial resolution or with integral light from the entire visible disk. These have usually involved continuous observations of various spectral lines by optical resonance techniques.

Fifteen years after the discovery of 5-min oscillations by Leighton¹⁷ came the first incontrovertible observations of global solar pulsations by Deubner¹⁸ who succeeded in resolving the diagnostic κ - ω diagram (or spatiotemporal power spectrum) into discrete lines. The periods were ~ 5 min and the horizontal wavelengths typically $1-3 \times 10^4$ km corresponding to spherical harmonics with high l values (typically a few hundred). These results subsequently proved that solar seismology is an exceedingly powerful diagnostic tool for investigating the solar interior¹⁹. The modes discovered by Deubner have provided information on layers located several thousand kilometres below the photosphere. The detection of lower-order modes is required for probing greater depths.

Thus, there is general agreement over the occurrence of solar pulsations with periods in the range of ~ 5 min. In fact, a recent study has resolved some low-angular (l), high-radial (n) overtones into discrete lines of amplitudes $0.1-0.3$ m s⁻¹ separated by an average uniform spacing of 67.8 μ Hz (ref. 20). However, for various reasons (including possible solar noise, interference

by the Earth's atmosphere and other local considerations and the severe problems in conducting these difficult observations at normal mid-latitude locations), the validity of reports of longer period global solar pulsations, especially one at 2 h 40 min (refs 21, 22), has not been universally accepted²³⁻²⁵.

The polar solar observatory

The advantages of undertaking certain types of astronomical observations at the geographic South Pole became apparent²⁶ during a program of cosmic ray research in Antarctica²⁷. More recently, we recognized that the South Pole enabled us to pursue the solar oscillation problem in conditions that cannot be duplicated anywhere else on Earth:

- (1) during the austral summer, the Sun remains essentially at a constant angular altitude, thereby eliminating the difficulties introduced by the day-night cycle;
- (2) uninterrupted data runs appreciably longer than those attainable elsewhere are feasible, thus significantly improving both the ω -resolution and the potential for detecting long-period oscillations;
- (3) the Amundsen-Scott Station at the South Pole is, in fact, at an elevation corresponding to a pressure altitude of $\sim 3,400$ m;
- (4) the atmosphere is colder than elsewhere, hence the precipitable water vapour content is exceedingly low, and the transparency is good, providing extended periods of coronal seeing;
- (5) the polar plateau constitutes an absolutely uniform terrain (a desert of snow) out to great distances in all directions;
- (6) the large trend due to the Earth's rotation, which can never be removed as perfectly as is required, is absent at the planet's rotational axis.

Consequently, an American-French programme was organized to conduct this experiment during the austral summer 1979-80. Only the main characteristics of the apparatus constructed for obtaining the required observations in the hostile polar environment ($T \leq -30$ °C) will be summarized here.

A sodium optical resonance photoelectric spectrophotometer developed at Nice (France) for full disk measurements of the Doppler shifts in the 5,896 Na D1 line²⁸ was modified to operate in the rigorous conditions at the South Pole. A vertical telescope (8 cm, $f/18$), specifically designed and constructed for this experiment at Bartol, provided two nonrotating solar images, one of which was focused on the guiding sensor array, the other on the spectrophotometer.

Extensive photography in HLy α during the preceding austral summer²⁹ revealed that the telescope completely satisfied the requirements for the present programme.

We attempted to create the best conditions possible by eliminating all potential man-made sources of spurious effects such as the local source of thermal turbulence arising from the

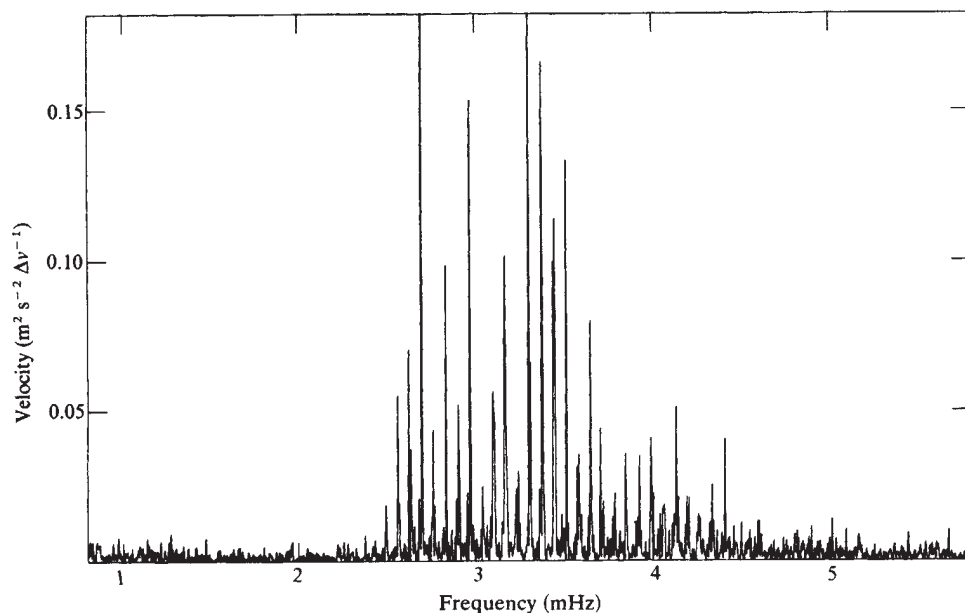


Fig. 1 Power spectrum of the continuous 5-day full-disk Doppler shift measurements recorded at the South Pole from 31 December, 1979 to 5 January, 1980. The resolution of the power in the 3-mHz range into many discrete equidistant lines separated by $68 \mu\text{Hz}$ indicates that global p -modes corresponding at least to l values of 0 and 1 are observed. Note that the small peaks around 2.4 mHz represent global oscillations with an amplitude $<10 \text{ cm s}^{-1}$ corresponding to motion of the solar radius $<5 \text{ m}$, or $7 \times 10^{-6} \text{ arc s}$.

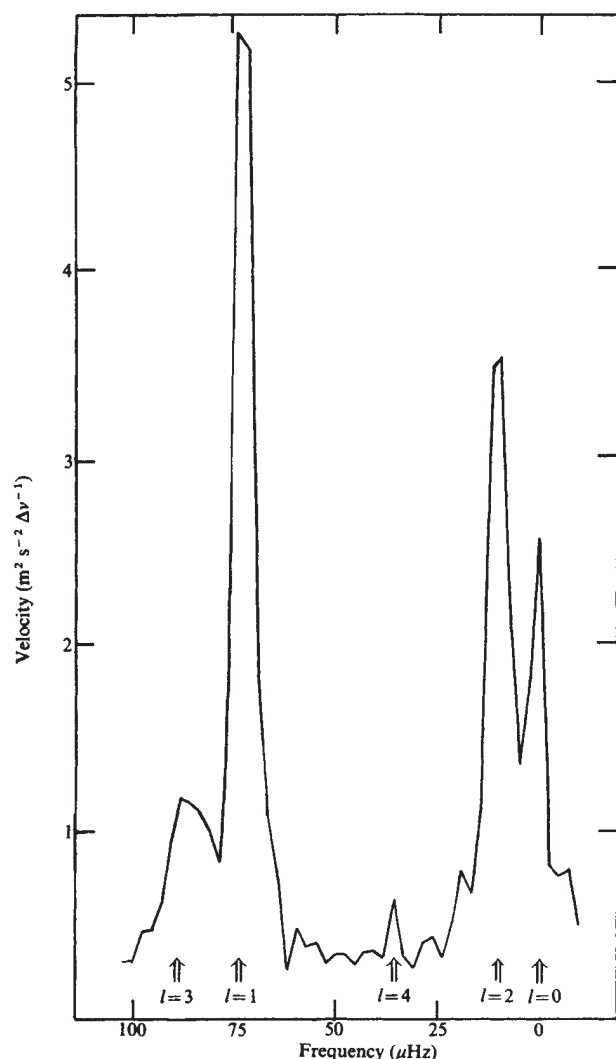


Fig. 2 A superposed frequency analysis of the frequency range between 2.4 and 4.8 mHz reveals the average shape of spectral lines displayed in the power spectrum of Fig. 1. The horizontal axis indicates where the theory predicts the positions of $l = 1, 2, 3$ and 4 modes if the one on the extreme right is assumed to be $l = 0$. The good agreement leaves no room for doubt. Note that the natural width of each separate mode indicates a Q value of the order of 600.

Amundsen-Scott station complex which would be seen at the same time every day, a site 8.1-km upwind from the base was selected. There, the well insulated $3.5 \text{ m} \times 2.5 \text{ m}$ laboratory building mounted on a sled was installed in a deep trench. The wanigan was then enclosed by a plywood roof and vestibule walls fore and aft, and was buried in the snow. The telescope and instrument package were mounted on the surface 30 m away. A 300-m cable connected the observatory to the diesel-powered electrical generator positioned downwind with respect to the prevailing wind direction. The exhaust emerged practically at ambient temperature, and neither the generator nor the track vehicle utilized for transport produced any detectable atmospheric disturbance.

Observations

Historical meteorological records and experience gained during 1978–79 made us hope for one 5-day period of uninterrupted optimal seeing during December–January. In fact, this did materialize, and the 5-day interval was extended to ~ 7 days by remaining on the air during two brief cloudy periods, which did not interrupt the tracking. In addition, clean data (cloud free) were recorded during intervals of additional hours ranging from 5 to 10, yielding high-quality tape recordings covering more than 200 h.

Data analysis and results

The definitive results of the analyses so far completed use part of the available data—basically the 5-day continuous run. The results mostly concern: (1) whether the widely discussed oscillation of period 160 min could be confirmed in the ideal conditions that prevail at South Pole; and (2) the investigation of the theoretically expected line spectrum of the 5-min oscillation.

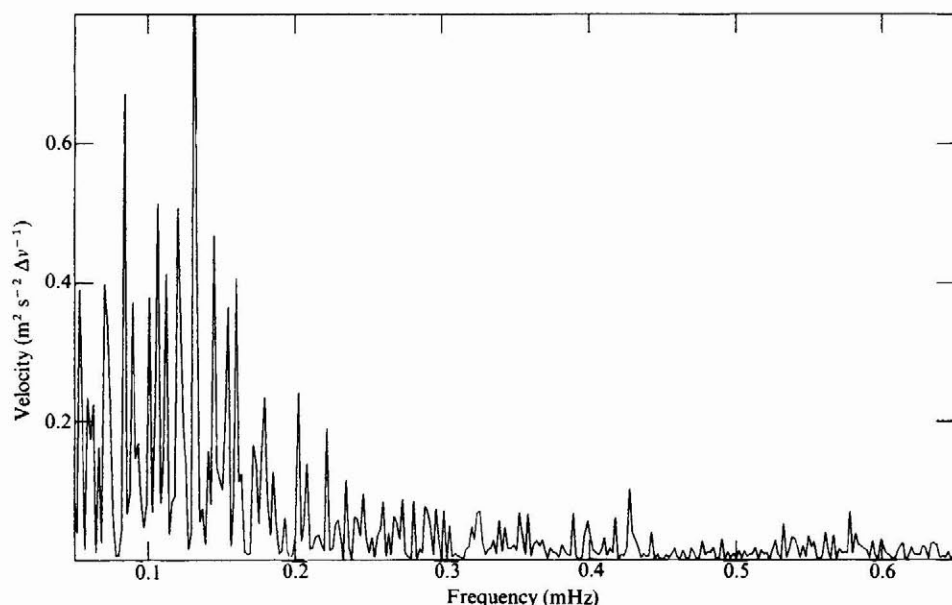
Note that the noise level at all frequencies was significantly lower than has ever been attained previously, by about an order of magnitude.

The 5-min range

Figure 1 shows the power spectrum of the 5-day data sample. It reveals that the power in the 3-mHz range is resolved into many equidistant peaks, separated by $68.0 \mu\text{Hz}$. This confirms the result published by Isaak's group²⁰. This constant displacement of $68 \mu\text{Hz}$ clearly indicates that modes of low degree with both odd and even values of l are observed, because the frequency separation between modes of high order with the same value of l is expected to be around $130\text{--}145 \mu\text{Hz}$ (depending on the theoretical model)³¹.

Modes with values of l between 0 and 3 may possibly contribute substantially to the observed line spectrum. Each line

Fig. 3 The low-frequency part of the power spectrum shown in Fig. 1. Its spiked structure is consistent with statistical departure around a broad, continuous spectrum. A spiked spectrum of oscillatory g -modes, a broad-band solar convective spectrum as well as a broad-band telluric spectrum could separately be responsible for this result.



would then contain contributions from modes with even l or odd l with almost coincident frequencies. The frequency separation between neighbouring modes with $l = 0$ and $l = 2$, and with $l = 1$ and $l = 3$ is expected to be $\sim 10 \mu\text{Hz}$ and $16 \mu\text{Hz}$ respectively³¹. Indeed, many peaks in Fig. 1 seem to be split into two or more components. To verify the significance of this splitting, we have used the equidistance to obtain the average shape of odd and even peaks by a 'superposed frequency analysis' of the spectrum between 2.4 and 4.8 μHz for an interval equal to 136.0 μHz . Figure 2 shows the result of this analysis, and clearly indicates that all modes between $l = 0$ and $l = 3$ and possibly even $l = 4$ contribute to the power spectrum. It is quite reassuring that the $l = 3$ modes display a significantly lower amplitude due to the smearing effect of the full disk integration.

In addition to the identification of the spherical harmonics associated with the spectral peaks, another important result emerges from Fig. 2. The width of the four main peaks indicates a Q value of about 600, or a typical damping time of 2 days. Indeed, if shorter data samples, of, say, 12 h, are Fourier analysed, the energy present in a given spectral peak does not seem to be constant in time or regularly modulated as would be expected from overstable beating pulsations. Rather, an individual peak seems to be suddenly excited and then slowly damped over a 2-day interval, unless it is re-excited before this time. A more complete statistical analysis of this temporal behaviour, embracing the total amount of data, will be necessary, but it is already clear that these modes around the 5-min period have a damping time of about 2 days and are spasmodically excited.

The intermediate frequency range

Figures 1 and 2 show the asymptotic behaviour of low degree global modes that must be predicted by solar models. Between 2.4 and 5 mHz the order n (the number of nodes along a solar radius in the vertical component of the displacement eigenfunction) must typically be between ~ 15 and 35 for these peaks³¹. To be able to identify each peak with one given order would require the detection of p modes with longer periods, and if possible all modes until the fundamental, the period of which is expected to be of the order of 1 h. Figure 1 shows that peaks with approximately uniform separation can be followed, with the limit of noise, towards low frequencies below 1 mHz. The same type of superposed frequency analysis, described above, over the range 0.8–2.4 mHz, demonstrates that $l = 0$, $l = 1$ and $l = 2$ normal modes may be present in this frequency interval, with a mean amplitude of less than $3\text{--}5 \text{ cm s}^{-1}$. Comparing this information with theoretical model predictions, one can be assured that, for example, the first peak which emerges significantly above the noise level near 2.23 mHz must be an

$l = 0\text{--}2$ pair with $n = 15 \pm 1$. To suppress the indicated uncertainty of the n -number definitely, the first four or five spherical harmonics must be identified, and this is more difficult because the asymptotic frequency equidistance prediction is no longer valid in the frequency range 0.3–0.8 mHz. We hope to be able to eliminate this ambiguity eventually by including the totality of recorded data.

The long period range

For periods longer than 1 h, we obtain an increasing power spectrum with decreasing frequency. Figure 3 shows this long-period power spectrum, for periods between roughly 30 and 300 min. Its spiked structure is consistent with pure statistical fluctuations around a broad, continuous curve. Although it may contain some solar information from oscillatory gravity wave or broad-band convective spectrum, this result alone does not allow us to conclude that any spectral peak is characteristic of a long-period solar oscillation.

The 160-min period

The presence of the 160-min solar oscillation originally discovered in the Crimea and observed in phase coherence during the past few years both in the Crimea and at Stanford was looked for using a superposed epoch analysis of the data processed thus far (5 days). Figure 4 shows the result of this analysis, together with the sine wave which would be expected by extrapolating the phase and amplitude according to the average of the Crimean

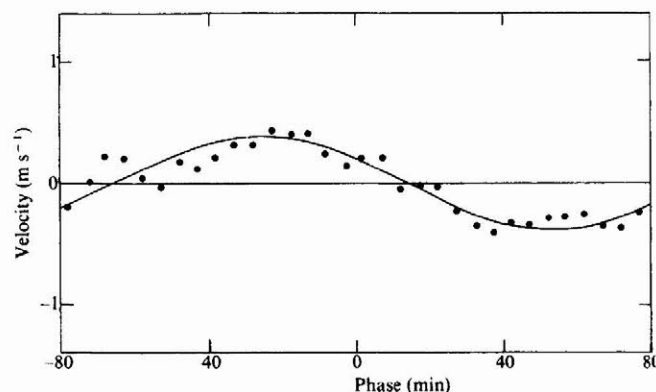


Fig. 4 The superposed epoch analysis of a data sample extending over 5 days (45 periods of 160 min). The points represent the South Pole data, and the solid line is the average based upon the observations obtained at the Crimean Observatory and Stanford.

and Californian results. The present result seems to be consistent with the latter. However, note that following a recent study of error analysis of superposed epoch procedure by Forbush *et al.*³⁰, standard methods of assigning statistical uncertainties may be not valid in this case.

Discussion

As we have analysed only part of the data processed so far, it would be premature to undertake a comprehensive discussion of theoretical implications concerning, for example, the depth of the convective zone, helium abundance, or other solar parameters. We have, therefore, only presented observational results which can be regarded as definitive:

- (1) Although the 160-min oscillation, in phase with the wave expected from the latest Russian and American results, has been detected, further analysis is required to determine its significance.
- (2) The power in the 5-min range is resolved into many equidistant peaks separated by 68 μHz . The splitting of these peaks,

which is different for even and odd lines, indicates that global modes with l values of 0, 1, 2 and 3 have been identified.

- (3) These global solar oscillations are not overstable, but are spasmodically excited, with a damping time of about 2 days ($Q = 600$).

- (4) Comparison between theoretical and observed frequencies of a large number of different modes has made it possible to reduce the uncertainty in the specification of the order of the spherical harmonics to unity.

These results are very encouraging for future astronomical research at the South Pole.

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1. Mazzitelli, J. *Astr. Astrophys.* **79**, 251 (1979).
2. Davis, R. Jr, Evans, J. C. & Cleveland, B. T. *Proc. Conf. Neutrinos 53* Lafayette (1978).
3. Gough, D. O. *2nd Assemblée Générale Européenne de Physique Solaire*, 81 (CNRS, Paris, 1978).
4. Musman, S. & Nye, A. N. *Astrophys. J. Lett.* **212**, L95 (1977).
5. Livingston, W. D., Milkey, R. & Slaughter, C. *Astrophys. J.* **211**, 281 (1977).
6. Beckers, J. M. & Ayres, T. R. *Astrophys. J. Lett.* **217**, L69 (1977).
7. Deubner, F. L. *Astr. Astrophys.* **57**, 317 (1977).
8. Hill, H. A. & Stebbins, R. T. *Astrophys. J.* **200**, 471 (1975).
9. Hill, H. A., Stebbins, R. T. & Brown, T. M. *Atomic Masses and Fundamental Constants Vol. 5*, 622 (Plenum, New York, 1976).
10. Brown, T. M. thesis, Univ. Colorado (1977).
11. Brown, T. M., Stebbins, R. T. & Hill, H. A. *Astrophys. J.* **223**, 325 (1978).
12. Hill, H. A. & Caudell, T. P. *Mon. Not. R. astr. Soc.* **186**, 327 (1979).
13. Fossat, E., Harvey, J. W., Hausman, M. & Slaughter, C. *Astr. Astrophys.* **59**, 279 (1977).
14. KenKnight, C., Gatewood, G. D., Kipp, S. L. & Black, D. *Astr. Astrophys.* **59**, L27 (1977).
15. Clarke, D. *Nature* **274**, 670 (1978).
16. Fossat, E., Grec, G. & Harvey, J. W. *Astr. Astrophys.* (in the press).
17. Leighton, R. B. *Nuovo Cimento Suppl.* **22** (1961).
18. Deubner, F. L. *Astr. Astrophys.* **44**, 371 (1975).
19. Deubner, F. L., Ulrich, R. K. & Rhodes, E. D. *Astr. Astrophys.* **72**, 177 (1979).
20. Claverie, A., Isaak, G. R., McLeod, C. P., Van der Raay, H. B. & Roca Cortes, T. *Nature* **282**, 591 (1979).
21. Severny, A. B., Kotov, V. A. & Tsap, T. T. *Nature* **259**, 8 (1976).
22. Scherrer, P. M., Wilcox, J. J., Kotov, V. A., Severny, A. B. & Tsap, T. T. *Nature* **277**, 635 (1979).
23. Fossat, E. & Grec, G. *2nd Assemblée Générale Européenne de Physique Solaire*, 151 (CNRS, Paris, 1978).
24. Grec, G. & Fossat, E. *Astr. Astrophys.* **77**, 351 (1979).
25. Worden, S. D. & Simon, G. W. *Astrophys. J. Lett.* **210**, L1 (1976).
26. Willer, A. A. *Polar Research, A Survey*, 170 (National Academy of Sciences, Washington DC, 1970).
27. Pomerantz, M. A. *Antarctic Research Series Vol. 29*, 12 (American Geophysical Union, Washington DC, 1978).
28. Grec, G., Fossat, E. & Vernin, J. *Astr. Astrophys.* **50**, 221 (1976).
29. Kusoffsky, U. & Pomerantz, M. A. *Proc. IAU General Assembly*, Montreal (1979).
30. Forbush, S. E., Pomerantz, M. A. & Duggel, S. P. (in preparation).
31. Christensen-Dalsgaard, J. & Gough, D. O. *Nature* **288**, 544–547 (1980).

Is the Sun helium-deficient?

J. Christensen-Dalsgaard* & D. O. Gough†

* Institut d'Astrophysique, Université de Liege, 4200 Cointe Ougrée, Belgium

† Institute of Astronomy, and Department of Applied Mathematics and Theoretical Physics, University of Cambridge, Silver Street, Cambridge CB3 9EW, UK

The recent observations of solar 5-min oscillations of low degree agree approximately with the predictions of a standard solar model with normal abundances of helium and heavy elements. Much of the apparent discrepancy noticed when the observations were first announced was a result of having neglected the influence of the Sun's atmosphere in the normal mode analysis of the theoretical models. Our standard solar models are not in perfect agreement with observation, but it seems that major modifications will not be necessary to remove the remaining small discrepancies.

CLAVERIE *et al.*'s¹ announcement of the resolution of a sequence of peaks in the power spectrum of whole-disk Doppler observations of the Sun, with frequencies in the range 2–4 mHz, produced an immediate discussion of the implications regarding the Sun's internal structure². The peaks were interpreted as being the result of groups of p modes of high order, of alternately odd and even degree². Direct comparison of the observed mean spacing between the peaks with predictions from a sequence of three solar models having different internal compositions³ gave precise agreement only when the internal abundances Y and Z of helium and heavy elements were rather low ($Y \approx 0.19$, $Z \approx 0.004$). A similar result seemed to be true of a sequence of models studied earlier by Iben and Mahaffy⁴, although in this case it was necessary to extrapolate the composition. However, the discrepancy between observation and the 'standard' models, with $Z = 0.02$ and $Y \approx 0.25$, was $< 2\%$, and without careful consideration of the uncertainties in both theory and observation it could not be concluded that the Sun is necessarily deficient in helium and heavy elements.

Nevertheless, there has already been some discussion of the implications of such a postulate⁵.

The observations of Grec *et al.*⁶ have confirmed the original results and have provided us with more accurate and detailed knowledge of the oscillation frequencies. We report here a further theoretical study of the 5-min modes, in the adiabatic approximation, and compare the results with the new observations. Although agreement is not perfect, we find no reason to conclude that the abundances Y and Z are surprisingly low.

Observations

Both Claverie *et al.* and Grec *et al.* measured the shift in a spectrum line in integrated sunlight, and the time dependence of the shift was analysed. In particular, power spectra of the data revealed almost uniformly spaced peaks in the range 2–5 mHz. Similar observations had been made before^{7–9}, and although there is a strong hint of discrete peaks in the results of Grec and Fossat⁸, they were never unambiguously resolved, partly because the periods of observation were too short¹⁰.

The most accurate observations are those of Grec *et al.*⁶. These were made at the South Pole, where the Sun can be seen continuously for long periods of time. The results have not yet been fully analysed, but a power spectrum of 120 h of continuous data is available. In agreement with previous observations^{1,7-9,11}, there is a concentration of power near 3 mHz. Moreover, almost all that power is contained in discrete peaks, whose mean spacing in the range 2.4–4.7 mHz is half of 136.0 μ Hz.

Interpretation of the observations

We accept that the direct cause of much of the observed power is an oscillation of the Sun that can be represented well by a superposition of linear normal modes. Because the observations integrate light from the entire solar disk, the measurements are sensitive only to modes of low degree. Consequently the modes are probably *p* modes of high order, because no solar *f* or *g* mode of low degree has a frequency as high as 2 mHz.

The frequencies of adiabatic *p* modes of low degree *l* and high order *n* are given approximately by¹²

$$\nu_{n,l} \approx (n + \frac{1}{2}l + \varepsilon)\nu_0 - [l(l+1) + \delta]A\nu_0^2\nu_{n,l}^{-1} \quad (1)$$

where

$$\nu_0 = \left(2 \int_0^R c^{-1} dr\right)^{-1} \quad (2)$$

and ε , δ and A are other constants of the equilibrium solar model. Here c is the adiabatic sound speed in the equilibrium model, r is a radial coordinate and R is the radius of the Sun. The approximation was derived assuming that $l \ll n$, and for such modes it provides a good representation of the numerical calculations¹⁰. The second term is a relatively small correction to the first. It therefore follows that sequences $\nu_{n,l}$ with fixed l and varying n are approximately uniformly spaced with separation close to ν_0 , that two such sequences with different l have almost coincident frequencies provided both values of l are either odd or even, and that the frequencies of modes with odd l lie approximately midway between those of modes with even l . The frequencies thus fall into almost uniformly spaced groups, which we label with $i = 2n + l$.

Theoretically one would expect modes with high n and low l to be excited to amplitudes that on average are roughly independent of l at fixed $\nu_{n,l}$, and there is some observational evidence that this is so¹⁰. Consequently each peak in the observed power spectra should contain contributions from several modes with almost identical frequencies. Thus the value of 136.0 μ Hz reported by Grec *et al.*⁶ for twice the mean spacing between the peaks in their power spectrum represents approximately the spacing between a sequence of eigenfrequencies at fixed l and varying n . As l increases, so does the second term in equation (1), which destroys the correspondence between frequencies of modes with different l . However, once l is large enough for the divergence to be substantial, the sensitivity of the observations is too low for it to matter. Indeed, theoretical estimates¹⁰ suggest that the observed signal is dominated by contributions from only those modes with $l \leq 3$, and that the expectation $\bar{\nu}_i$ of the peak frequencies is approximately

$$\bar{\nu}_i = \begin{cases} 0.51 \nu_{n,0} + 0.49 \nu_{n-1,2} & i \text{ even} \\ 0.87 \nu_{n,1} + 0.13 \nu_{n-1,3} & i \text{ odd} \end{cases} \quad (3)$$

The observations of Grec *et al.*⁶ confirm this interpretation. It has been possible to resolve structure in the major peaks, which presumably arises from the presence of modes with slightly different frequencies. To reduce noise and the effects of interference, Grec *et al.*⁶ performed a superposed frequency analysis on the spectrum, by dividing the spectrum into segments of length 136.0 μ Hz and adding the segments of spectrum. The result is shown in their Fig. 2. Although this procedure does not take account of the nonlinearity of the dependence of $\nu_{n,l}$ on n , the separation of each peak into two main components is clear and the relative power within each of those components is roughly in accordance with theoretical predictions.

Oscillation eigenfrequencies and the influence of the atmosphere

Oscillation eigenfrequencies of the solar models discussed in ref. 3 and some models of Christensen-Dalsgaard¹³ have been computed as described in ref. 10. The most important difference between these computations and those discussed in ref. 3 is that here some account has been taken of the solar atmosphere.

The influence of the atmosphere is important only when the oscillation frequency ν is comparable with Lamb's¹⁴ acoustical cutoff frequency $\nu_c = \gamma g/4\pi c$, which is the minimum frequency at which acoustic waves can propagate vertically in the atmosphere. Here γ is the adiabatic exponent ($\partial \ln p / \partial \ln \rho$), where p , ρ and g are pressure, density and specific entropy, and g is the gravitational acceleration. In the solar atmosphere, $\nu_c \approx 5$ mHz. Provided $\nu < \nu_c$, *p* modes are reflected by an evanescent region that includes the atmosphere, and are thus confined to the solar interior. The lower the frequency, the deeper the level at which reflection takes place. Thus when $\nu \ll \nu_c$, reflection occurs so deep in the Sun that the modes are almost unaffected by the presence of the atmosphere. Neglecting the atmosphere in the computations reported in ref. 3, for example, introduces errors in the frequencies stated explicitly in that paper that are no more than about a part in 10^5 .

When ν is comparable with ν_c , the inertia of the atmosphere is an appreciable fraction of the total mass above the reflecting region. In this case the atmosphere introduces a phase shift in the spatial oscillation of the eigenfunction, and so changes the frequency. It is only when ν is quite close to ν_c , however, that the detailed structure of the atmosphere is important. Otherwise the atmosphere moves almost hydrostatically, and its influence on the phase shift depends only on its mass, which is directly related to the equilibrium photospheric pressure.

We substantiate the discussion above by considering a plane-parallel polytropic layer under constant gravity, supporting an isothermal atmosphere of the same composition. The polytrope is taken to have the structure

$$p = p_0 z^{m+1}, \quad \rho = \rho_0 z^m, \quad z_{ph} \leq z \leq Z \quad (4)$$

where z is a vertical coordinate measured downwards, and p_0 , ρ_0 and m are constants. The isothermal atmosphere in $(-\infty, z_{ph})$ is determined by requiring that temperature and pressure, and hence density, are continuous at the photosphere $z = z_{ph}$.

The horizontal wavelengths of the solar modes of low degree are much greater than the scale height in the atmosphere. Therefore, to determine the influence of the atmosphere on the eigenfrequencies, we need only consider purely vertical oscillations. We assume a rigid base at $z = Z$, and select those modes with an energy density that tends to zero as $z \rightarrow -\infty$. Thus we are ignoring the potential influence of the corona, or nonlinearities in the upper atmosphere¹⁵⁻¹⁷.

The analysis is a straightforward generalization of that presented by Lamb¹⁴. The eigenfrequencies ν_n of high order n , satisfying $2\pi n \gg m^2$, are given by

$$\nu_n \approx (n + \frac{1}{2}m - \frac{1}{4})\nu_0 - \frac{(4m^2 - 1)\nu_0^2}{8\pi^2\nu_n} - \frac{\nu_0}{\pi} \tan^{-1} \Phi_m(\alpha_n, \lambda_n), \quad (5)$$

where

$$\Phi_m(\alpha, \lambda) \equiv \frac{J_{m+1}(\lambda) - \alpha J_m(\lambda)}{Y_{m+1}(\lambda) - \alpha Y_m(\lambda)}$$

$$\lambda_n = (m+1)x_n, \quad \alpha_n = x_n^{-1} - (x_n^{-2} - 1)^{1/2}, \quad x_n = \nu_n/\nu_c \quad (6)$$

and the characteristic frequency ν_0 is now defined by

$$\nu_0^{-1} = 2 \int_0^Z \left(\frac{\rho_0}{\gamma p_0 z} \right)^{1/2} dz; \quad (7)$$

J_m and Y_m are Bessel functions of order m . If the isothermal atmosphere were replaced by a rigid lid of the same mass, the

Table 1 Properties of solar models

Model	Opacity	M	Z	Y	D (10^5 km)	$\Delta\nu$ (μ Hz)	$\nu_{16,1}$ (mHz)	$\nu_{28,1}$ (mHz)	$\nu_{n,0} - \nu_{n-1,2}$ (μ Hz)	$\nu_{n,1} - \nu_{n-1,3}$ (μ Hz)
A	CS/L	200	0.02	0.251	1.67	136.6	2.404	4.041	10	16
B	CS/L	200	0.004	0.187	1.20	134.8	2.353	3.970	13	17
C	CS/L	200	0.001	0.160	0.29	129.9	2.336†	3.895†	13	18
A*	CS/L	200	0.02	0.251	1.67	138.1	2.412	4.069		
1	CT/S	201	0.02	0.247	1.97	136.7	2.417	4.056		
2	CT/S	401	0.02	0.247	1.97	136.5	2.416	4.053		
3	CT/L	201	0.02	0.253	1.91	136.5	2.415	4.053		
4	CRS/S	201	0.02	0.249	1.75	135.9	2.409	4.039		
H	CT/S	201	0.02	0.266	1.42	136.5	2.398	4.035		

Y and Z are the initial abundances by mass of helium and heavy elements; D is the present depth of the convection zone. CS refers to the tables of Cox-Stewart²³, CRS to Carson (unpublished, but see ref. 24) and CT to Cox-Tabor²⁵; the L or S refers to linear or spline interpolation in the tables. M is the number of mesh points between the centre and the photosphere of the model. Electron screening was taken into account in computing nuclear reaction rates only in models 1–4 and H. An evolutionary calculation was not performed for the chemically homogeneous model H; the initial helium abundance of that model was estimated by homology scaling from its present value of 0.303. The mean frequency separation $\Delta\nu$ is twice the mean separation, in the range 2.3–4.1 mHz, between the peaks in the power spectrum of an ensemble of all the modes. The mean separations between neighbouring $l=0$ and $l=2$ eigenfrequencies and between neighbouring $l=1$ and $l=3$ eigenfrequencies of models A, B and C are listed in the last two columns, and are averaged over the same frequency range as $\Delta\nu$. The mean separation between neighbouring $l=0$ and $l=4$ eigenfrequencies of model A in that range is 35 μ Hz. The expectations of the relative power in the peaks of a power spectrum produced by modes of different degree can be obtained from ref. 10. These are 0.56, 1.0, 0.54, 0.06 and 2×10^{-3} for $l=0, 1, 2, 3$ and 4 respectively. Frequencies of dipole modes with frequencies near 2.4 and 4.0 mHz are listed in columns 8 and 9. For model C these frequencies† are $\nu_{17,1}$ and $\nu_{29,1}$.

eigenfrequencies would still be given by equation (5), but with $\alpha_n = \frac{1}{2}x_n$. If the atmosphere were ignored entirely, and the lagrangian pressure perturbation were assumed to vanish in the photosphere, then $\alpha_n = 0$. Replacing the atmosphere by a polytrope of index m reduces the equilibrium model to a complete polytrope; in that case the last term in equation (5) is removed, yielding what is essentially Lamb's¹⁴ result. Note that in the limit of small x_n , $\alpha_n \approx \frac{1}{2}x_n$ and the leading terms in the expansions of $J_{m+1}(\lambda_n)$ and $\alpha_n J_m(\lambda_n)$ cancel in equation (6). Thus to leading order, the isothermal and polytropic atmospheres and the rigid lid all yield the same eigenfrequencies.

The result of ignoring the atmosphere entirely is to overestimate the eigenfrequencies by

$$\delta\nu_n = \pi^{-1}\nu_0[\tan^{-1}\Phi_m(\alpha_n, \lambda_n) - \tan^{-1}\Phi_m(0, \lambda_n)] \quad (8)$$

When x_n is small this reduces to

$$\delta\nu_n \approx \nu_0[\Gamma(m+1)\Gamma(m+2)]^{-1}(\frac{1}{2}\lambda_n)^{2(m+1)} \quad (9)$$

where Γ is the gamma function, which shows the increasing importance of the atmosphere as the frequency rises.

We can use equation (8) to obtain a rough estimate of the error in the mean frequency separation introduced by ignoring the atmosphere. If the mean is evaluated between ~ 2 and 4.5 mHz, and $m \geq 2$, the result is $\sim 1 \mu$ Hz, and depends only weakly on m and the precise frequency range over which the mean is evaluated. This estimate is comparable with the difference between the mean separation originally obtained by Claverie *et al.*¹ and the mean that can be deduced from equations (3) and the values quoted for the standard solar model in ref. 3. This justifies our repeating the theoretical eigenfrequency analysis with the atmosphere included.

Equation (8) cannot be used to correct the frequencies in refs 3 and 4, because the Sun is neither plane parallel nor polytropic. It is easy to show that even though the solar atmosphere is thin compared with its radius, in the limit $x_n \rightarrow 0$ a geometrical correction is introduced into equation (9) which is of the same order as the value derived here. Indeed, the difference between our new results and those of ref. 3 obey a power law when $\nu \leq 2$ mHz, with exponent $2(m+1) \approx 8.6$, but with a coefficient considerably greater than that given by equation (9). For higher values of ν , the numerical results and the plane-parallel theory agree more closely.

Mean frequency separation of modes

The mean frequency separation between groups of modes was computed in the range 2.3–4.1 mHz, using equation (3) to determine the mean peak positions. The frequency range was chosen to correspond approximately to that within which

Claverie *et al.*¹¹ could resolve discrete peaks. The mean separations between the peaks with odd l and with even l were computed separately; in all the cases we investigated the two values differed by no more than 2 parts in 10^4 . The values in Table 1 are averages of the two values and therefore represent twice the mean spacing of the peaks in a power spectrum of an ensemble of all the oscillations.

Model A was computed in the standard way from the zero-age main sequence. In models B and C, which have low initial abundances of heavy elements, the surface was assumed to have been contaminated continuously by accreted material during evolution, at a rate chosen to give $Z=0.02$ throughout the convection zone at present. In all cases an atmosphere in hydrostatic equilibrium with the T - τ relation of the Harvard-Smithsonian Reference Atmosphere¹⁸ out to $\tau=10^{-4}$ was added, as described in ref. 10. The outer boundary condition—that the lagrangian pressure perturbation vanishes—was applied at $\tau=10^{-4}$. Included for comparison is model A*, which is simply model A with the outer oscillation boundary condition applied at the photosphere, as in ref. 3.

Four other so-called standard models are included in Table 1, to give some idea of the sensitivity of the results to uncertainties in the theory. These are models 1–4 of ref. 13. They all have $Z=0.02$, and differ from each other through the use of different opacity tables, or a different number of mesh points. Electron screening was taken into account in computing the nuclear reaction rates, which was not the case for models A, B and C, and a more accurate numerical scheme was used.

Comparison of theory with observation

Given a sequence of solar models, one can attempt to find by interpolation a model which best fits the data. First, the frequency separation is interpolated, which should give sufficient information to identify the modes, and then the frequencies themselves can be used.

The original attempt to do this² took account of only the frequency separation, because the observed frequencies were not available¹. Interpolation was essentially between models A, B and C, except that the oscillation boundary condition had been applied at the photosphere. This overestimated the mean theoretical separations by $\sim 1.5 \mu$ Hz for models A and B and 1μ Hz for model C, and led to the tentative conclusion that a model close to model B was preferred. However, the reduction of the theoretical eigenfrequencies that has resulted from including the atmosphere, and the slight upward revision of the estimate of the actual frequency separation by the South Pole measurements⁶, have now brought the standard model into closer agreement with observation. Interpolation with respect to

frequency separation alone suggests a model between models A and B, but closer to model A.

The superposed frequency analysis of Grec *et al.*⁶, summarized in their Fig. 2, enables us to identify the modes. By comparison of the relative power within the peaks and the peak separations with the theory summarized in ref. 10 and Table 1, it is evident that the peaks on the left result from modes of odd degree and those on the right from modes of even degree. Note that there is even a hint of the $l = 4$ modes about 35 μHz below the radial modes. Thus it has been possible to identify the peaks in the power spectrum of the observations at, for example, $\nu = 2.426$ and 4.057 mHz, as modes with $l = 1$ (E. Fossat, personal communication); and we deduce that these modes are of the order of $n = 16$ and 28 respectively. Note, however, that the frequencies of these modes cannot be reproduced by interpolation between models A and B. Extrapolation beyond the standard model to one slightly richer in helium and heavy elements reduces the discrepancies, but perfect agreement is not possible.

Other solar models

It is not possible to fit all the observations with the eigenfrequencies of a model interpolated in or extrapolated from the sequence A, B, C. However the variations amongst models 1–4 seem to be sufficiently great to suggest that agreement could be achieved with a model that does not differ substantially from our standard. That does not necessarily imply that such a model is a good representation of the Sun. There may be other quite different models which could reproduce the observed frequencies equally well.

In particular, we consider the transient mixed model that was discussed by Dilke and Gough¹⁹ as a potential explanation for terrestrial glaciation and the low solar neutrino flux. Unfortunately we do not have sufficient information to calculate eigenfrequencies, but we do have the integral in equation (2) evaluated between $r = 0$ and the radius in the radiative interior within which 98% of the mass is contained. Though ν_0 is not equal to the frequency separation $\Delta\nu$, we have found in other models that its variation follows that of $\Delta\nu$ quite closely. Therefore we have estimated ν_0 for the transient models as follows.

We selected three values of the time t . The first, t_1 , was 4,700 Myr, immediately before a phase of mixing; the second, t_2 , was 2 Myr later, which is near to the luminosity minimum; and the third, t_3 , was 5 Myr after t_1 , which is roughly when the luminosity and radius first return to values close to those at t_1 . At each time the radiative interior was scaled homologously, varying Y at fixed Z by such an amount as to bring the model to the current solar luminosity. Model envelopes with the solar photospheric radius and the same composition as the unburnt region of the scaled interior were then fitted to the interior, by insisting that the mass and temperature be continuous at the fitting radius. A further small homologous scaling was then necessary to bring the total mass of the entire model to the solar value. The remainder of the integral in equation (2) could then be computed.

During the transient mixing phase of a single model, the luminosity and radius vary. The decrease in temperature, and consequently sound speed, outweighs the smaller relative

decrease in radius, and causes ν_0 to decrease. However, when three different models each having the same luminosity and radius at the different epochs t_1 , t_2 and t_3 are compared, the reverse is true. The values of ν_0 at t_2 and t_3 were found to be 1.2 and 0.7% greater than the value at t_1 . We conclude that the frequency separation $\Delta\nu$ has a similar variation.

As a second example we consider a chemically homogeneous solar model in thermal balance, model H. We computed this model to see whether g modes of low degree and low order could have periods close to 160 min. This particular model, whose properties are summarized in Table 1, has modes $g_2(l = 1)$ and $g_4(l = 2)$ both with periods close to 150 min, and a relatively minor change of parameters could no doubt change them to 160 min. The low degree 5-min p mode eigenfrequencies are hardly distinguishable from those of model A.

Conclusion

Approximate agreement between the eigenfrequencies of a standard solar model and the observations of Grec *et al.*⁶ has been found in the frequency range 2.3–4.1 mHz. Thus there seems to be no evidence from the whole-disk observations of 5-min oscillations that the solar structure is very different from that of a standard model. Moreover, models with low abundances of helium and heavy elements are incompatible with observation.

The frequencies discussed here contain enough information to differentiate between the models in Table 1 and the real Sun. Therefore we know that none of our models is exact. Small changes in the physics could possibly improve the agreement. In particular, our equation of state may be too inaccurate. Moreover, there have been revisions in opacities and nuclear reaction rates since the models were computed²⁰, and when these are taken into account the discrepancies may disappear. Nevertheless, the relative insensitivity of the frequencies to considerable changes in structure, as illustrated by the properties of the mixed models, has suggested that agreement might also be achieved with models rather different from the standard.

To differentiate between the models it may be necessary to seek other measures. For example, only those models with convection zones as deep as $\sim 2 \times 10^5$ km are compatible with observations of the 5-min oscillations of high degree^{21,22}. Thus the chemically homogeneous model H seems to be ruled out. Another quantity that might be measurable is the buoyancy frequency N beneath the convection zone which controls the g mode frequencies. Unfortunately, no solar g mode has yet been identified. However, there is some influence of N on the p modes of low degree, especially those that are of low order and which do seem to be present in the whole-disk power spectrum⁶. The data currently available are too noisy to determine the frequencies of the low-order modes precisely, but further analysis of the data obtained by Grec *et al.*⁶, and possibly longer observations of the same type, may make it possible. Then we shall be in a better position to judge the models.

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- Claverie, A., Isaak, G. R., McLeod, C. P., van der Raay, H. B. & Roca-Cortés, T. *Nonradial and Nonlinear Stellar Pulsation* (eds Hill, H. A. & Dziembowski, W. A.) 181–183 (Springer, Heidelberg, 1980).
- Christensen-Dalsgaard, J. & Gough, D. O. *Nonradial and Nonlinear Stellar Pulsation* (eds Hill, H. A. & Dziembowski, W. A.) 184–190 (Springer, Heidelberg, 1980).
- Christensen-Dalsgaard, J., Gough, D. O. & Morgan, J. G. *Astr. Astrophys.* **73**, 121–128; **79**, 260 (1979).
- Iben, I. Jr & Mahaffy, J. *Astrophys. J. Lett.* L39–L43 (1976).
- Isaak, G. R. *Nature* **283**, 644–645 (1980).
- Grec, G., Fossat, E. & Pommerantz, M. *Nature* **288**, 541–544 (1980).
- Fossat, E. & Ricort, G. *Astr. Astrophys.* **43**, 243–252 (1975).
- Grec, G. & Fossat, E. *Astr. Astrophys.* **55**, 411–420 (1977).
- Dittmer, P. H. *Astrophys. J.* **224**, 265–275 (1978).
- Christensen-Dalsgaard, J. & Gough, D. O. *Mon. Not. R. astr. Soc.* (in the press).
- Claverie, A., Isaak, G. R., McLeod, C. P., van der Raay, H. B. & Roca-Cortés, T. *Nature* **282**, 591–594 (1979).
- Tassoul, M. *Astrophys. J. Suppl.* **43**, 469–490 (1980).

- Christensen-Dalsgaard, J. *Mon. Not. R. astr. Soc.* (in the press).
- Lamb, H. *Proc. Lond. Math. Soc.* **7**, 122–141 (1909).
- Rosenwald, R. D. & Hill, H. A. *Nonradial and Nonlinear Stellar Pulsation* (eds Hill, H. A. & Dziembowski, W. A.) 404–412 (Springer, Heidelberg, 1980).
- Logan, J. D., Hill, H. A., Puccio, P. & Rosenwald, R. D. *Nonradial and Nonlinear Stellar Pulsation* (eds Hill, H. A. & Dziembowski, W. A.) 413–428, (Springer, Heidelberg, 1980).
- Gough, D. O. *Nonradial and Nonlinear Stellar Pulsation* (eds Hill, H. A. & Dziembowski, W. A.) 273–299 (Springer, Heidelberg, 1980).
- Gingerich, O., Noyes, R. W., Kalkofen, W. & Cuny, Y. *Solar Phys.* **18**, 347–365 (1971).
- Dilke, F. W. W. & Gough, D. O. *Nature* **240**, 262–264; 293–294 (1972).
- Bahcall, J. N. *et al. Phys. Rev. Lett.* **45**, 945–948 (1980).
- Lubow, S. H., Rhodes, E. J. Jr & Ulrich, R. K. *Nonradial and Nonlinear Stellar Pulsation* (eds Hill, H. A. & Dziembowski, W. A.) 300–306 (Springer, Heidelberg, 1980).
- Berthomieu, G. *et al. Nonradial and Nonlinear Stellar Pulsation* (eds Hill, H. A. & Dziembowski, W. A.) 307–312 (Springer, Heidelberg, 1980).
- Cox, A. N. & Stewart, J. N. *Astrophys. J. Suppl.* **19**, 243–279 (1970).
- Carson, T. R. A. *Rev. Astr. Astrophys.* **14**, 95–117 (1976).
- Cox, A. N. & Tabor, J. E. *Astrophys. J. Suppl.* **31**, 271–312 (1976).

The constancy of the solar diameter over the past 250 years

John H. Parkinson*, Leslie V. Morrison† & F. Richard Stephenson‡

* Mullard Space Science Laboratory, University College London, Holmbury St Mary, Dorking, Surrey RH5 6NT, UK

† Royal Greenwich Observatory, Herstmonceux Castle, Hailsham, East Sussex BN27 1RP, UK

‡ Department of Geophysics, University of Liverpool, Liverpool L69 3BX, UK

Reconsideration of meridian circle observations with analysis of transits of Mercury and durations of total solar eclipses indicate that there has been no detectable secular change in the solar diameter during the past 250 yr.

ALTHOUGH the output of energy by the Sun is generally assumed to be produced entirely by nuclear processes in the core, experiments to detect the neutrino flux yield only about one-third of the expected value. Among the many ingenious suggestions that have been made to resolve this apparent discrepancy¹ is the recent claim that observational evidence from about 1840 suggests that the Sun's radius is shrinking at the considerable rate of about 0.1% per century². The gravitational energy released in this contraction of the Sun, or at least of the outer layers, would then supplement the Sun's luminosity from nuclear reactions.

The brightness of the Sun depends principally on the core temperature so that, in contracting models of the Sun, the core temperature would need to be reduced to bring the predicted brightness of the Sun into agreement with observation. Such a change would also lower the predicted neutrino emission, possibly to the extent of being consistent with the observed rate. This would remove a major stumbling block in modern astrophysics. Only a small fraction of the Sun's mass could be involved in such a gravitational contraction as a uniform contraction of the entire Sun at the rate suggested would increase the luminosity to 10^2 – 10^3 times its present value.

Here we investigate the reality of the observed contraction of the solar diameter and show that it is based on a misinterpretation of the meridian circle observations used. A more careful analysis, combined with two other methods of investigation, show that there has been no detectable secular change in the Sun's diameter since at least AD 1700.

Meridian circle measurements

One of the earliest tasks of the Royal Observatory at Greenwich was to measure the position of the Sun in the sky. Using a meridian circle, the times at which the leading and following edges crossed the meridian were noted and the angles from the zenith to the upper and lower limbs were measured. By averaging these pairs of measurements the Sun's position could be obtained, whereas subtraction gives the apparent horizontal and vertical solar diameters.

Analyses of meridian transit measurements for periodic changes in the diameter have produced inconclusive results³. There have, however, been reports of secular trends found in the measurements; for example, a horizontal semi-diameter decrease of 0.01 arcs per year from 1890 onwards has been reported⁴. Recently Eddy and Boornazian² claimed to find a

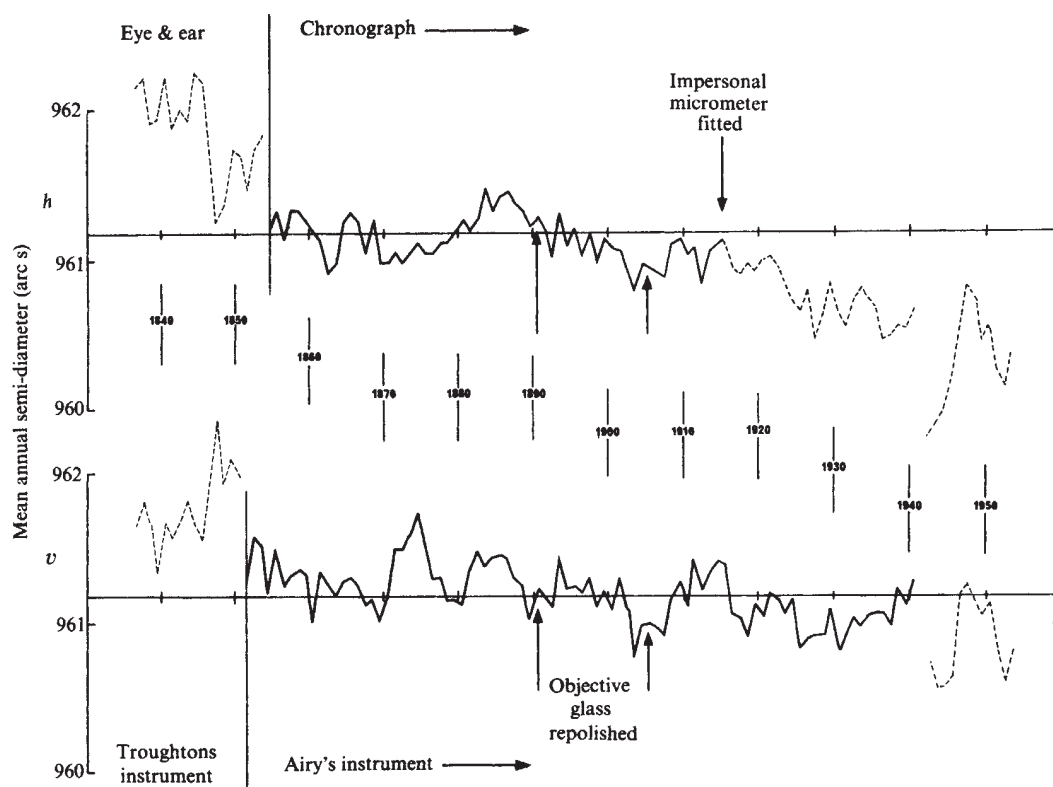


Fig. 1 Annual mean values of the Sun's horizontal (h) and vertical (v) semi-diameters derived from meridian circle observations made at Greenwich². The most reliable results are joined by solid lines and the less reliable ones by dashed lines.

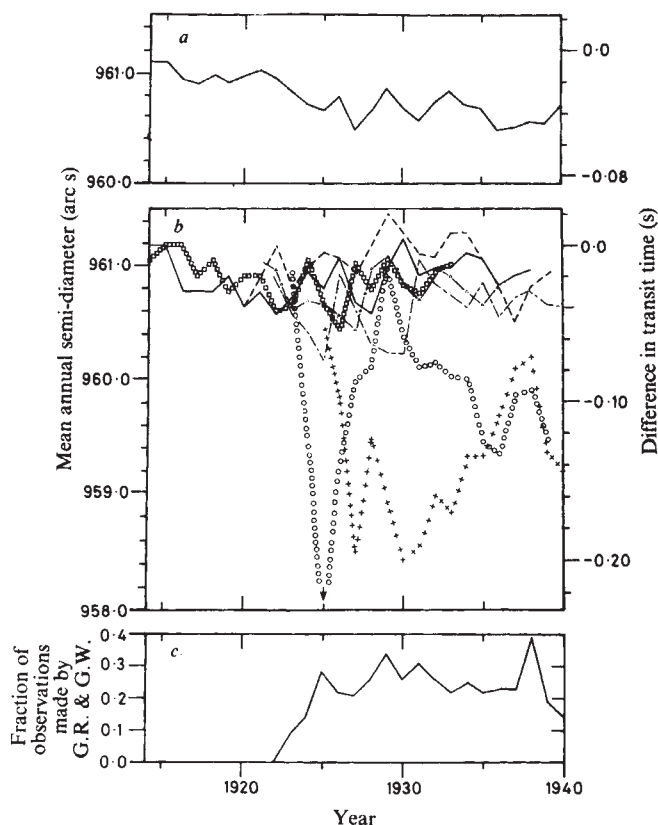


Fig. 2 *a*, Mean horizontal semi-diameter for the seven regular observers between 1915 and 1940 showing an apparent decrease of 0.01 arcs per year. *b*, Individual annual means for the seven regular observers. Five observers: W, $\square$; H.A., —; R.C., ---; L.S., — — —; H.B., $\triangle$ give consistent results, whereas G.R., $\circ$ and G.W., + show erratic changes. *c*, The fraction of measurements made each year by G.R. and G.W. As the fraction increased, so the semi-diameter seemed to decrease.

similar trend in the horizontal diameter and about half that rate in the vertical diameter, the evidence for both trends commencing in 1836. As such a significant change would have profound implications for the evolution of the Sun, it warrants further study using other techniques.

Such analyses must be complicated by the instrumental changes at Greenwich since 1836. In 1851, 'Airy's telescope' replaced Troughton's instrument⁵. Figure 1 shows the variation in the annual mean horizontal and vertical semi-diameters derived by Eddy and Boornazian. There is a clear discontinuity in the measurements of the vertical semi-diameter coinciding with the change of telescope showing that the measured

diameter depends crucially on the instrument used. After Airy's instrument had been in service for only one year, it was stated that "no other instrument... can be compared with this for steadiness and accuracy of adjustments or for the accuracy of results"⁶.

As the horizontal measurements were made by noting the length of time taken for the Sun to cross a wire in the eyepiece of the telescope, the measured horizontal diameter depends on both the clarity of the image formed and the accuracy with which the transit time could be measured. Before 1854 the observer watched the Sun while listening to the ticking of a pendulum clock. The precise time at which the Sun crossed the meridian was then interpolated mentally between two successive ticks. In 1854 this crude 'eye and ear' method was replaced by a chronograph, which had the advantage of recording the time automatically. Again, after only one year the observations were judged to be "... indisputably of a higher order of excellence than those formerly made by the ordinary method of eye and ear"⁷. The horizontal semi-diameters in Fig. 1 show a clear discontinuity when the observing method was changed.

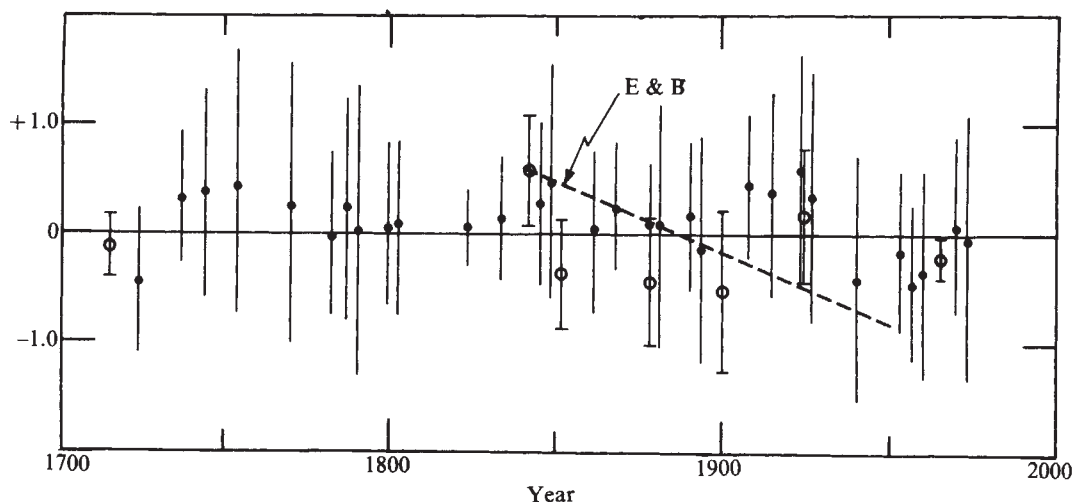
The telescope objective glass was repolished in 1891 and again in 1906 (ref. 8) which may have altered the definition of the image and hence the apparent diameter of the Sun. In 1915 the 'impersonal micrometer' was fitted and again this may have affected the horizontal diameter measurements. Observations with Airy's telescope ceased in 1953 when the reversible transit instrument came into operation at Herstmonceux.

A high degree of skill and dexterity were required to operate Airy's meridian circle, and, from its early days, the influence of observer bias was recognized^{3,9-11}. In particular, Thackeray demonstrated that each observer had a personal bias which was maintained consistently throughout his observing career. For the nine observers in the period 1861-83, the range of career means was as much as 4.8 arcs for the vertical diameter and 2.2 arcs for the horizontal diameter.

Generally, such personality effects would be expected to increase the scatter in the results, as happens for the data after World War II when as many as 14 observers, most with little or no previous experience, made measurements. The period from the introduction of the impersonal micrometer to 1940 shows a decrease of ~ 0.02 arcs yr^{-1} in the mean horizontal semi-diameter. During this time there were seven regular observers, five of whom reported self-consistent annual means with no apparent trends. The other two observers exhibited strong, erratic personal biases, as shown in Fig. 2. As the number of observations made by these two individuals increased through the early 1920s, so the resultant mean diameter based on all the observers decreased, giving a spurious trend.

The observational reports from Greenwich show that weather and seeing conditions were often far from ideal with many comments such as 'through cloud', 'unsteady', 'ill defined', 'poor image', 'limbs boiling', 'definition extremely bad', and so on.

Fig. 3 Apparent changes in the semi-diameter of the Sun (arcs) deduced from transits of Mercury ($\bullet$) and total solar eclipses ($\circ$). The zero point corresponds to 959.63 arcs. The half-bar lengths are the standard deviations of the distributions, the standard errors of the means lie in the range ± 0.1 to ± 0.2 arcs. The dashed line was deduced by Eddy and Boornazian² from horizontal meridian circle observations in the period 1836-1953 and has been adjusted for irradiation.



These grave difficulties show that meridian circle observations are unsuitable for investigating possible changes in the solar diameter. We now discuss other, more accurate methods which have the advantage of covering a longer time span and are not so dependent on the defects of the instrument used.

Transits of Mercury

Observations of the time taken for the planet Mercury to transit across the face of the Sun provide one of the most accurate methods of detecting long-term changes in the solar diameter. These observations are spread over some 300 yr. Due to the particular geometry of the orbits of the Earth and Mercury, transits occur only in May or November, and there are ~14 in a century. The maximum duration of a central transit in May is ~8 h and in November it is ~6 h. Thus, if the instants at which Mercury seemed to touch the rim of the Sun were timed to a precision of 1 s, the resolution obtained for the solar diameter would be ~0.1 arcs. However, due to the observational difficulty of discerning when Mercury first touches the limb of the Sun, the standard deviation of the observations of each transit are typically in the range 0.5–1.0 arc s. It is also necessary to restrict observations to timings of the first and last instants (contacts 2 and 3) at which Mercury appears completely inside the limb of the Sun; these internal contacts are more definite than the external ones.

Over 2,000 timings of the internal contacts of 30 transits of Mercury spread over the past 250 yr have been used in this analysis. The observations of these transits have been collected by Morrison and Ward^{12,13} mainly to determine fluctuations in the Earth's rate of rotation and the relativistic advance in the perihelion of Mercury's orbit. When allowance has been made for these and other orbital parameters (see solution 2, Table III of ref. 13), the angular separation between the centres of the Sun and Mercury, calculated from the observed times of internal contact, should be equal to the difference of their semi-diameters.

The residual angular separation for each observation has been calculated using values of 959.63 and 3.37 arc s for the semi-diameters of the Sun and Mercury at unit distance. The means and standard deviations of the distributions of the residuals for 30 of the transits in the period 1723 to 1973 are plotted in Fig. 3 where they are compared with the meridian circle trend claimed by Eddy and Boornazian². To make this comparison we have subtracted 1.55 arc s from the meridian circle observations, this being the generally accepted value used to account for the somewhat arbitrary effect of irradiation¹⁴.

The results from the transits shown in Fig. 3 are at variance with the change in the semi-diameter claimed by Eddy and Boornazian between 1836 and 1953 and rule out any extrapolation of this trend to other epochs. A linear regression analysis of the Mercury transits produces a secular trend of -0.14 ± 0.08 arcs per century in the solar semi-diameter, broadly agreeing with Shapiro¹⁵, who reported a decrease of less

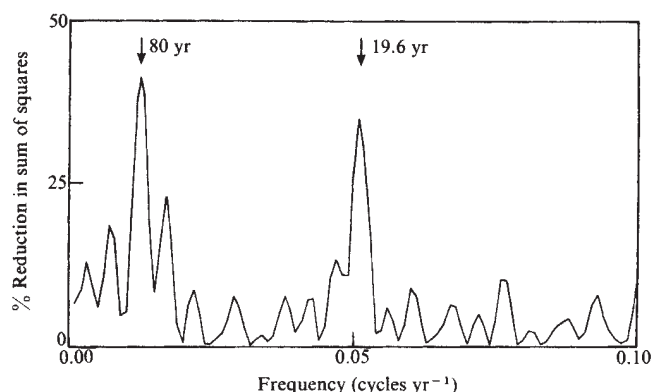


Fig. 4 Spectrum of the apparent changes in the semi-diameter deduced from transits of Mercury shown in Fig. 3.

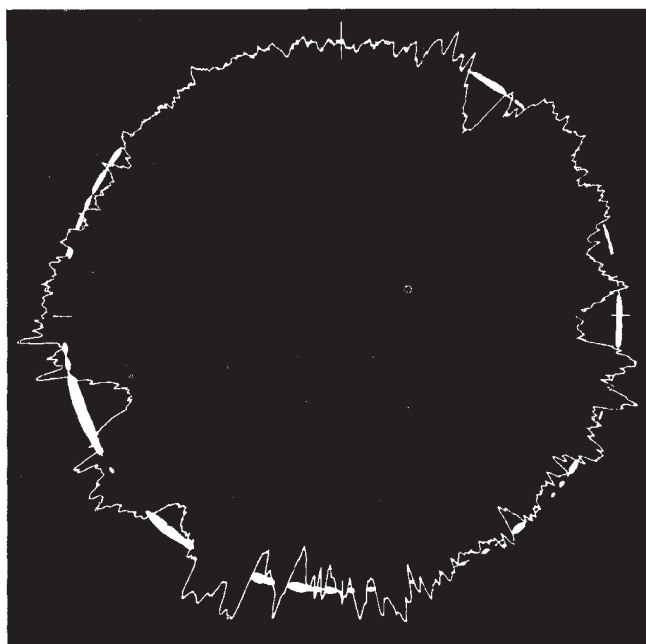


Fig. 5 An enlargement of a frame from J. H. Mathers' 16-mm cine film of the solar eclipse of 20 May 1966 taken on the island of Lesbos in Greece. The jagged outline of the Moon, exaggerated by a factor of 60, is shown superimposed on the frame. Bailey's beads can be seen in the lunar depressions.

than 0.15 arcs per century with >90% confidence from a smaller set of Mercury transit observations.

We have also examined the data for possible periodic changes using a least-squares frequency analysis. Sine curves were fitted to the data by least squares and the percentage reduction in the sum of the squares is shown in Fig. 4. There are significant peaks at 80 yr and 19.6 yr. Removal of one of these sinusoids from the data reduces the other to the level of the noise. One is therefore the alias of the other, and is caused by the particular spacing of the transits. Because the 80-yr peak is the higher of the two, it is more likely to be the basic causal period, but we cannot be certain of this¹⁶.

The amplitude, phase and standard errors of the 80-yr sinusoid were found to be given by:

$$\begin{aligned} \text{change in semi-diameter (arc 2)} = \\ 0.24(\pm 0.08) \sin 2\pi[(t-1700)/80 + 0.43(\pm 0.04)] \end{aligned}$$

where t is the year of the observation.

Total solar eclipses

The observed durations of total solar eclipses, typically a few minutes, present an alternative method of detecting possible long-term changes in the solar diameter. The timing of such a duration to a precision of 1 s would give a measure of the Sun's semi-diameter to an accuracy of ~0.2 arcs assuming that the profile of the Moon were known accurately. In practice observations need to be restricted to the timing of the interval between the disappearance of the last and the reappearance of the first beads of light, which are seen at the irregular edge of the Moon at the beginning and end of total eclipses.

The edge of the solar photosphere is extremely well defined and eclipse observers have shown that, until the last bead of light shining down one of the depressions on the lunar limb has disappeared, then totality has not yet started. Perhaps the best test is the internal consistency of timings of totality by independent observers, which is typically a few seconds of time. Although the absolute times of the contacts might well be in error, only the duration of totality is important in determining the diameter and even in the early eighteenth century it was possible to time such durations to within a second or two by counting the beats of a pendulum clock.

In our investigation of solar eclipses, we have used visual observations of the durations of the total eclipses of 1715, 1842, 1851, 1878, 1900 and 1925. We have also analysed photographs of the eclipse of 1966, which was intermediate between total and annular.

After Halley¹⁷ had carefully organized timings of the 1715 eclipse, there are no comparable timings until 1842. From then until 1925, eclipses were chosen that were reasonably uniformly spaced in time and also the individual reports are fairly easily accessible. References are too numerous to cite here. After 1925 only scattered timings are available and we have accordingly ended our investigation of such material at that date.

In analysing these eclipse observations we have adopted the values of 959.63 and 932.58 arc s for the solar and lunar semi-diameters at mean distance and applied lunar limb profile corrections for each individual observation¹⁸, the errors of which are unlikely to exceed 0.2 arc s at any point. Many of the observations were made quite close to the central line of the shadow path, where the duration was greatest. We have rejected all observations for which the duration was less than ~1 min because the location would be very close to either of the edges of the zone of totality. In these circumstances it becomes difficult to identify exactly which depressions on the Moon's limb permit the appearance of the last and first beads of light. The results of this analysis are plotted in Fig. 3 alongside the Mercury transit data. For each eclipse, the mean correction to the Sun's semi-diameter and the standard deviation of the individual observations is shown.

During the eclipse of 20 May 1966, Mathers took a 16-mm cine film of the central phase from the island of Lesbos in Greece. From this point in the path of the eclipse, the calculated solar semi-diameter was only 0.07 arc s greater than that of the Moon. Mathers positioned himself almost exactly on the central line and at the time of maximum eclipse the Sun was seen to be

reduced to a broken circle of light composed of ~50 individual beads (see Fig. 5). On the eight frames nearest to the time of greatest phase we have been able to identify each bead of light with a depression of the lunar limb as plotted by Duncombe¹⁸. From a consideration of the visibility of the various beads, we deduce a correction of -0.22 ± 0.20 arc s to the adopted semi-diameter of the Sun. This provides a valuable additional point on Fig. 3.

Figure 3 shows that, in general, the accuracy of the eclipse results is similar to that of the Mercury transit data. If we include the eclipses with the transits in a linear regression analysis, the secular trend in the semi-diameter reduces to -0.08 ± 0.07 arc s per century. This corresponds to a change of $-0.008 \pm 0.007\%$ per century, well over an order of magnitude smaller than the rate claimed by Eddy and Boornazian.

Conclusion

The combined data sets of the Mercury transit and total solar eclipse observations, which cover a much longer time span than the material used by Eddy and Boornazian, demonstrate that there has been no detectable secular change in the solar diameter over the past 250 yr or so. However, there is some evidence that periodic changes of about 0.02% on a time scale of 80 yr may have occurred. The findings of Eddy and Boornazian are the result of instrumental and observational defects rather than real changes, hence the problem of the missing solar neutrinos still remains.

We thank Mr J. H. Mathers for use of his unique eclipse film. *Note added in proof:* In a recent preprint by Dunham *et al.*¹⁹ they deduce, from observations made near the limits of the total eclipses of 1715 and 1976–79, that the Sun's semi-diameter decreased by 0.34 ± 0.2 arc s in this interval. Their result depends critically on the interpretation put on the only observation made near the northern limit of the 1715 eclipse.

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1. Bahcall, J. N. *Space Sci. Rev.* **24**, 227–251 (1979).
2. Eddy, J. A. & Boornazian, A. A. *Bull. Am. astr. Soc.* **11**, 437 (1979); *Sci. News* **115**, 420 (1979); *Phys. Today* **35**, 17 (1979).
3. Gething, T. J. D. *Mon. Not. R. astr. Soc.* **115**, 558–570 (1955).
4. Gianuzzi, M. A. *Mem. Soc. astr. Ital.* **24**, No. 3 (1953).
5. Howse, D. *Greenwich Observatory Vol. 3*, 43–48 (Taylor & Francis, London, 1975).
6. *Mon. Not. R. astr. Soc.* **12**, 97 (1852).
7. *Mon. Not. R. astr. Soc.* **15**, 122–123 (1855).
8. Wittichell, W. M. *Occ. Not. R. astr. Soc.* **14**, 147–159 (1952).

9. Dunkin, E. *Mon. Not. R. astr. Soc.* **35**, 91–97 (1874).
10. Thackeray, W. G. *Mon. Not. R. astr. Soc.* **45**, 389–399, 464, 467–469 (1885).
11. Cullen, R. T. *Mon. Not. R. astr. Soc.* **86**, 344–349 (1926).
12. Morrison, L. V. & Ward, C. G. *R. Gr. Obs. Bull.* No. 181 (1975).
13. Morrison, L. V. & Ward, C. G. *Mon. Not. R. astr. Soc.* **173**, 183–206 (1975).
14. *Explanatory Supplement to the Astronomical Ephemeris*, 101 (HMSO, London, 1961).
15. Shapiro, I. I. *Science* **208**, 51–52 (1980).
16. Lomb, N. R. *Astrophys. Space Sci.* **39**, 447–462 (1976).
17. Halley, E. *Phil. Trans. R. Soc.* **29**, 245–262 (1715).
18. Duncombe, J. S. *U.S. Naval Obs. Circ.* No. 141 (1973).
19. Dunham, D. W. *et al. Science* (submitted).

Angiogenesis *in vitro*

Judah Folkman* & Christian Haudenschild

The Department of Surgery, Children's Hospital Medical Center and The Harvard Medical School and The Mallory Institute of Pathology, Boston, Massachusetts 02115

Cloned capillary endothelial cells, cultured in tumour-conditioned medium, form capillary tubes. By light and electron microscopy these tubes resemble capillaries in vivo. This first demonstration of angiogenesis in vitro: (1) shows that all the information necessary to develop an entire capillary network in vitro is expressed by one cell type; (2) suggests a mechanism for lumen formation; and (3) offers a possibility of distinguishing between direct and indirect angiogenesis factors.

MOST, if not all, solid tumours are capable of continuously inducing the proliferation of new capillary blood vessels from the host vascular bed. There is accumulating evidence that this property, angiogenesis, is important for the progressive growth of solid tumours^{1–3}. Previous studies have described the growth and regression of new capillaries *in vivo*^{4,5} but there is little understanding of how a capillary grows. It is not known how a lumen develops in a capillary sprout, how branches arise, or how

anastomoses are made between one capillary loop and another. Until now, angiogenesis could be studied only in animal models^{6,7} or in the chick embryo⁸. Lack of an *in vitro* model has made the investigation of angiogenesis as difficult as the study of blood coagulation would be without *in vitro* methods.

In a previous report, bovine capillary endothelial cells were cloned, and bovine and human capillary endothelial cells were carried in long-term culture⁹. We show here that in appropriate conditions, these capillary endothelial cells will form tubular networks *in vitro* which are almost identical, by light and electron microscopy, to capillary vascular beds *in vivo*.

* Present address: Children's Hospital Medical Center, 300 Longwood Avenue, Boston, Massachusetts 02115.

Fig. 1 Human capillary endothelial cells forming 'capillary' tubes (phase contrast). *a*, A cylindrical 'keyhole' vacuole (arrow) is the first evidence of tube formation. Capillary endothelial cells from Sertoli-Leydig ovarian tumour in a 16-yr-old girl. Third passage. $\times 115$. *b*, Same field 3 days later showing tubes that traverse the entire length of some cells, and connect with tubes of neighbouring cells. $\times 115$. *c*, Network of tubes formed by capillary endothelial cells from human foreskin. Tubes began to form 1 month after the primary cells were plated. $\times 115$. *d*, The junction of the capillary tube of one cell with another (arrow). String-like material within the lumen ('mandril') traverses the anastomosis between the two cells. $\times 230$. Capillary endothelial cells were isolated as described elsewhere⁹ except that 0.75% collagenase (Worthington type II) with 0.5% bovine serum albumin (BSA) was used for digestion of tissue. Human capillary endothelial cells were grown in tumour-conditioned medium⁹ mixed 1:1 with aortic-conditioned medium, 15% human serum and endothelial cell growth supplement (ECGS) 5.4 mg ml^{-1} (Collaborative Research). Aortic-conditioned medium was obtained from cloned bovine endothelial cells grown to confluence in T-75 flasks. Dulbecco's minimal essential medium (MEM) with 10% calf serum was changed every 48 h, centrifuged (2,000 r.p.m. for 10 min), passed through a Millipore filter (0.22 μm), and stored at -30°C . It was thawed and centrifuged immediately before use (A growth factor has been derived from aortic endothelial cells by Gajdusek *et al.*¹¹.) Human serum was heat-inactivated at 56°C for 30 min before use. It was convenient to make up the media before use in 50-ml polyethylene tubes (35 ml tumour-conditioned medium, 35 ml aortic-conditioned medium, 11.5 ml human serum and 15 mg ECGS). Unused media were stored at 4°C for a few days. Cultures were re-fed every 48 h. Bovine capillary cells will form capillaries optimally in a mixture of 60 ml tumour-conditioned medium + 30 ml aortic-conditioned medium + 10% calf serum and 15 mg ECGS. Occasionally bovine cells will form capillaries in tumour-conditioned medium mixed 1:1 with alpha MEM or with Dulbecco's MEM with 10% calf serum (especially late passage cells). Both bovine and human cells survive freezing in dimethyl sulphoxide (DMSO) and will form capillaries after thawing. Bovine cells have been carried in culture for up to 9 months and human cells for up to 5 months. During early passage, human capillary cells must pass at high density. When Falcon (3001) plates were used (for easier weeding of non-endothelial cells⁹) the capillary cells were plated into a glass cloning ring (6 mm i.d.) placed in the centre of the dish.

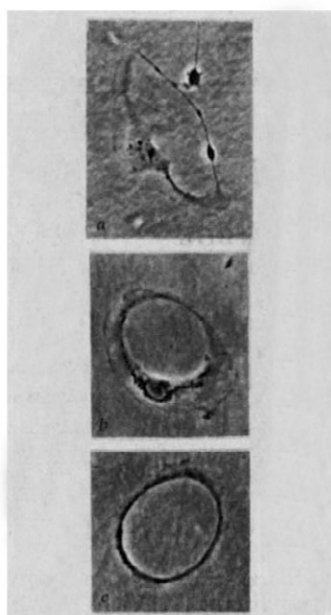
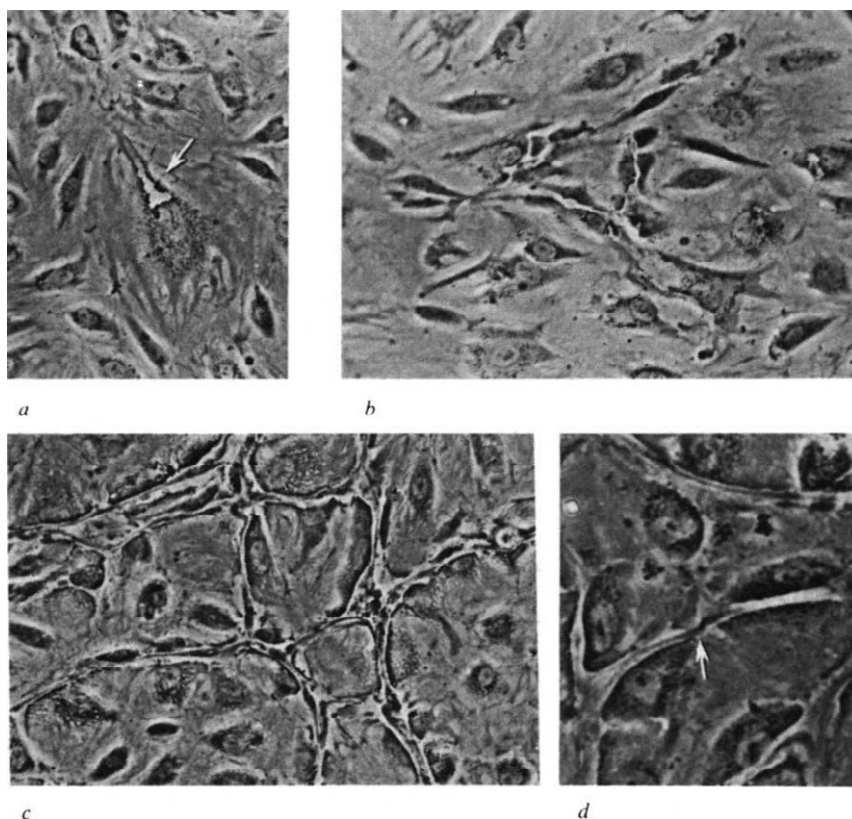


Fig. 2 Ring formation by single capillary endothelial cells (phase contrast). Human foreskin capillary endothelial cells without any neighbours, but stimulated by tumour-conditioned medium, seem unable to form a tube in three dimensions. Instead, they move cytoplasmic extensions along the bottom of the culture dish, until the tips of these extensions join to form a flat, two-dimensional 'ring'. *a*, *b* And *c* show different stages of this process. $\times 230$.

Isolation and culture of capillary endothelial cells

Capillary segments were isolated from bovine adrenal cortex, human foreskin, human spleen, an ovarian tumour, a neuroblastoma and a rhabdomyoma of the heart as described elsewhere⁹ (except for the modifications described in Fig. 1 legend). The amount of starting tissue need not be more than 0.1–0.5 g. Experience has shown that even a small tumour biopsy yields capillary segments in culture provided the biopsy is taken from the vascular, non-necrotic portion of the tumour. The capillary segments were cultured in tumour-conditioned medium on gelatin-coated plates⁹. The bovine cells were cloned, whereas endothelial cells from other tissues, also derived from a single capillary segment (2–4 capillary endothelial cells), were not cloned.

The evidence that these cultured cells are capillary endothelial cells, detailed elsewhere^{9,10}, is as follows: (1) each culture is initiated from one capillary segment of ~2–4 capillary endothelial cells. With experience, it is possible to distinguish capillary segments from arterioles or venules. (2) The capillary endothelial cells of different organs from the same animal and from different species seem to be almost identical by phase-microscopy. (3) Both human and bovine capillary endothelial cells contain factor VIII antigens⁹. (4) Cultured capillary endothelial cells have a similar ultrastructure to the capillary endothelial cells of their organ of origin: human cells contain Weibel-Palade bodies—bovine cells do not. (5) Both human and bovine capillary endothelial cells form 'rings' when they are in sparse culture (Fig. 2). (6) Early passage bovine capillary endothelial cells, and human capillary endothelial cells at any passage

require conditioned medium, and grow best in tumour-conditioned medium. However, endothelial cells from aorta and umbilical vein do not require conditioned medium for growth⁹. (7) Capillary endothelial cells increase their rate of migration in a dose-dependent manner when increasing concentrations of tumour-conditioned medium or tumour-derived factors are added¹⁰. In contrast, the migration of aortic endothelium is not affected by tumour-conditioned medium. (8) Capillary endothelial cells have a special matrix requirement (for example, gelatin⁹ or collagen¹¹) for their survival.

Development of capillary tubes *in vitro* and the origin of branches

When the endothelial cells of a single human capillary are allowed to proliferate, they form a small colony which enlarges gradually. When the colony reaches 3–5 mm, the centre approaches confluence. In ~50% of the cultures, capillary tubes begin to form in the middle zone of the colony, between the deeply confluent centre and the sub-confluent periphery. In

primary cultures of most human and bovine capillary cells, the earliest appearance of tubes is 20 days and the latest, 40 days. Capillary endothelial cells from a rhabdomyoma formed tubes 10 days after the primary culture was started. However, once tubes have formed in the primary culture, they appear earlier in subsequent passages. In the fourth passage, tubes developed in 5–10 days.

The first evidence of tube formation is the appearance (by phase contrast) of a cylindrical vacuole within an endothelial cell, which resembles a 'keyhole' (Fig. 1a). After 48 h, contiguous cells develop similar vacuoles which form a long tube connecting one cell to another (Fig. 1b). These tubes do not arise from cleavage planes between cells, but in fact traverse the cytoplasm, or the nucleus, so that the nucleus sometimes seems to be split into two parts (Fig. 1d). Electron micrographs taken at these locations always show a single nucleus which has a portion close to the lumen deformed into a thin plate positioned either at the bottom or the roof of the tube. Branches begin to appear after four or five cells have become connected, and within 5–10 days an entire network of tubes replaces the dense regions of the colony (Fig. 1c).

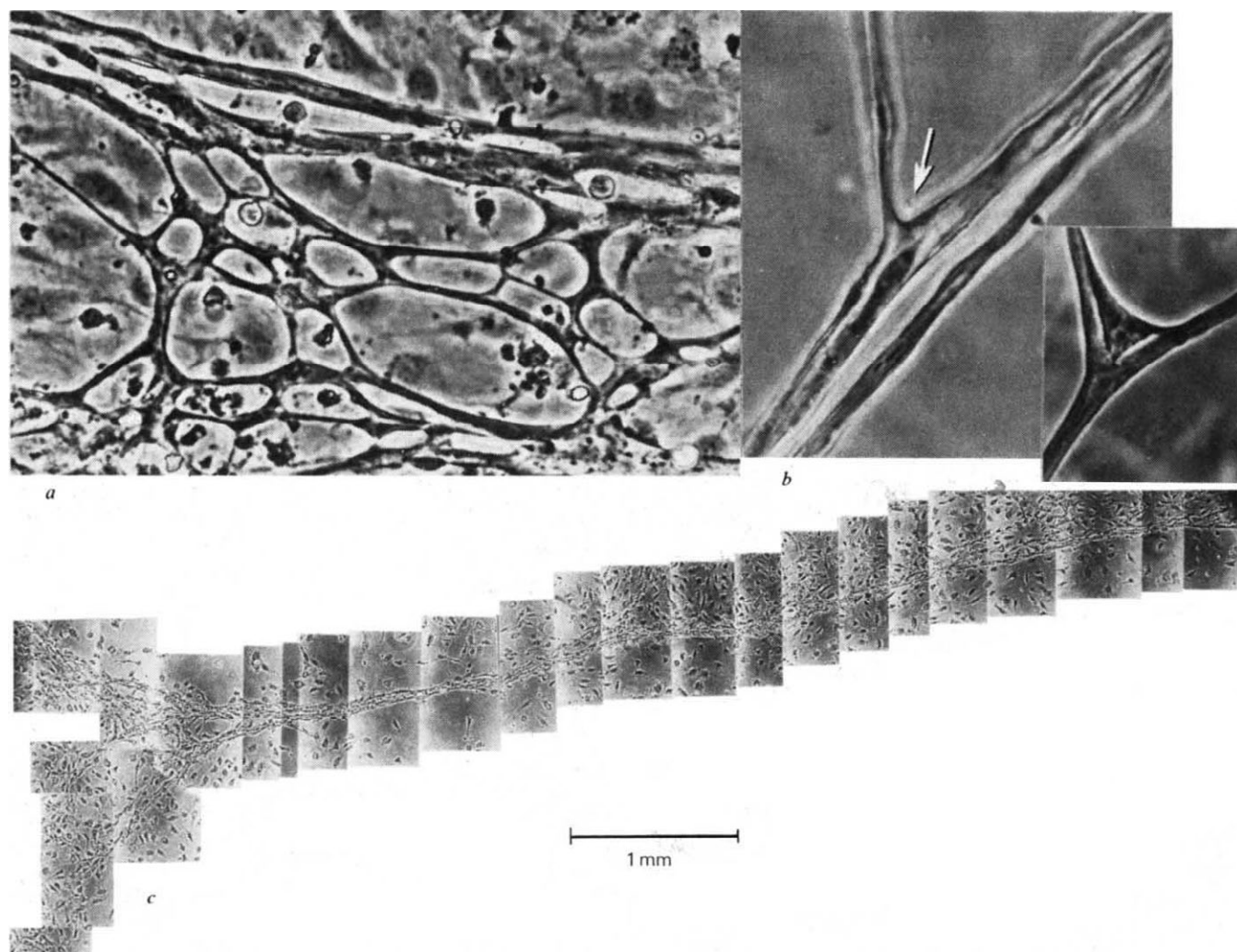


Fig. 3 Organization of a second layer of capillary tubes on the dorsal surface of the original monolayer of capillary endothelial cells (phase contrast). *a*, A network of tubes began to form in the original monolayer of these 11th-passage bovine capillary endothelial cells, after 2 weeks in the same plate. A branch that was directed out of the plane of the substratum led to the formation of a second layer of tubes. This photograph shows only the second layer in focus. The first cell layer (lying directly on the gelatin) is out of focus. $\times 115$. *b*, Branches formed *in vitro* by capillary endothelial cells derived from human foreskin. Note the position of the endothelial cell (arrow) that lies at the intersection of two limbs. Inset shows another branch bridged by the endothelial cell from which the branch was formed. These tubes formed in a second layer of capillary endothelial cells after they had been plated into the microwells of a gelatinized Cuprak dish. Monolayers formed in the wells that contained more than one cell. When tubes appeared in these monolayers, many branches were directed out of the plane of the substratum, presumably because of the curvature of the wells. The first layer is not visible here because it is out of focus. *c*, A capillary tube, 7.5 mm long, that grew *in vitro* from fourth-passage capillary endothelial cells from human foreskin.

A branch in the tubular network always originates within a single cell. Before a branch appears, the cylindrical vacuole within a cell forms a 'Y' or a 'T', each arm of which then aligns with the vacuole of another cell. An entire capillary network develops in this way. After a branch has matured, the original endothelial cell can usually be found at the intersection of three tubes (Fig. 3b). Occasionally, a branch will be directed out of the plane of the substratum—then a second layer of capillaries organizes on the dorsal surface of the cells that were originally plated. This three-dimensional growth results in a capillary network of multiple layers (Fig. 3a, c).

Remodelling and regression of capillary tubes

Time-lapse, phase contrast pictures (2 frames per min) taken continuously for 1 week, showed that established tubes undergo remodelling. New branches appear and others are contracted into the parent vessel within a period as short as 12 h. Once a tube has formed by the alignment of 5–10 endothelial cells, it presents a thin, phase-dense lining. The endothelial cells that embrace it also migrate along the tube and out to the tips of its branches. Tumour-conditioned medium has been shown to cause a 500% increase in migration of capillary endothelial cells *in vitro*¹⁰. Remodelling seems to be carried out primarily by migratory activity of capillary endothelial cells and not by mitosis. Furthermore, when cultures were incubated with ³H-thymidine (4 μ Ci ml⁻¹ for 48 h), endothelial cells lining the tubes were not labelled, but those at the periphery of the colony were.

When the cells in a capillary network were trypsinized and re-plated, new capillary tubes formed. There was no obvious difference between the capillary networks formed by capillary endothelial cells isolated from normal or malignant human tissue.

Ultrastructure of capillary tube formation

Transmission electron micrographs taken perpendicular and parallel to the plane of the culture dish prove that these aligned, phase-optically transparent structures are continuous lumina bordered by cell cytoplasm and membranes (plasmalemma), and that they form a network of tubes (Fig. 4a). The origin of a tube seems to be a true vacuole surrounded by one uninterrupted membrane (Fig. 4b). Later, the vacuole seems to become an extracellular tube surrounded by an extremely thin wall of cytoplasm of the endothelial cell. The tubular wall of one cell will connect to the tubular wall of another cell (Fig. 4c, d). Although we have been unable to observe the exact transition from an intracellular vacuole of one cell to an intercellular tube girdled by several cells, we have seen intermediate stages. For example, at one stage, the initially intracellular vacuole opens to form two cytoplasmic sheets (Fig. 5a) which are capable of joining with similar cytoplasmic extensions of a neighbouring cell, or with themselves (Fig. 5b). At these connections the structure resembles a junctional complex. (However, freeze-fracture and other studies to characterize these junctions formed *in vitro*, remain to be done).

Early in their development, the tubes are filled with a partially fibrillar, partially membranous and amorphous material of undetermined nature that apparently originates from the endothelial cells (Fig. 5c). In some areas, this material resembles basement membrane, and is also found outside the lumen in direct contact with the gelatin substratum. This material may serve as a guide or 'mandril' for the alignment of tubes from cell to cell. Later the material apparently dissolves, or is cleared from the lumen in some other way (Fig. 5a), because the tubes become hollow as the capillary network matures (Fig. 5d).

To control for the possibility that the tumour-conditioned medium and the gelatin-coated plates might induce tube

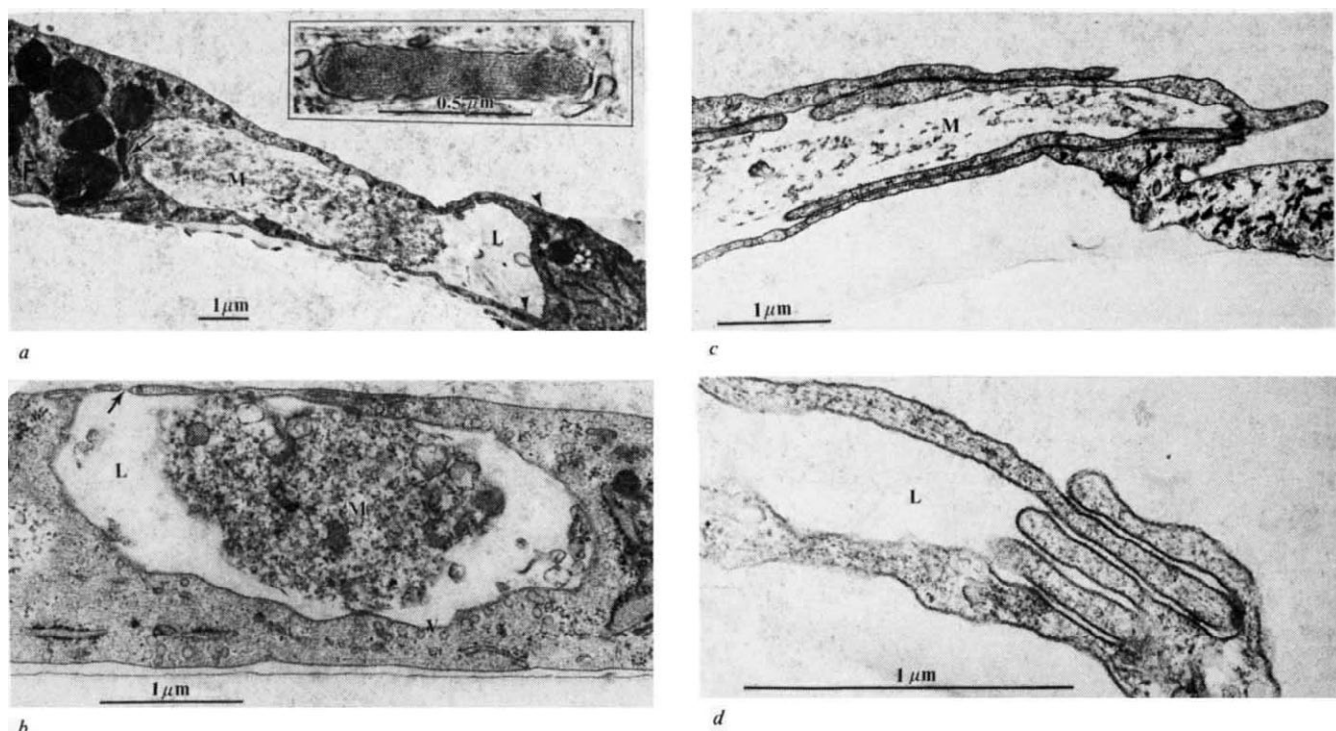


Fig. 4 Transmission electron micrographs of capillary formation. *a*, Micrograph taken in a plane perpendicular to that of the culture dish, showing the formation of a tube by human capillary endothelium. Note the amorphous and fibrillar material (M) within the lumen (L), the Weibel-Palade body (arrow), some myelinated figures (F) and the junction between the two cells (arrowheads). Insert shows higher magnification of Weibel-Palade body from another tube-forming human capillary endothelial cell. *b*, Earliest phase of tube formation within one cultured capillary endothelial cell derived from bovine adrenal. The micrograph is taken in a plane perpendicular to the culture dish and demonstrates the thin cytoplasmic plate which covers the lumen (L) and which shows structures resembling fenestrations (arrow). Note the intraluminal material (M) and the pinocytotic vesicles (V). *c*, Thin cytoplasmic plates surround the lumen of a tube formed *in vitro* by capillary endothelial cells derived from human foreskin. The intraluminal material (M) is disappearing. Micrograph taken in a plane perpendicular to the culture dish. *d*, Junctional complex formed *in vitro* by two capillary endothelial cells derived from an ovarian (Sertoli-Leydig) tumour. The lumen (L) is empty. Micrograph taken in a plane perpendicular to the dish.

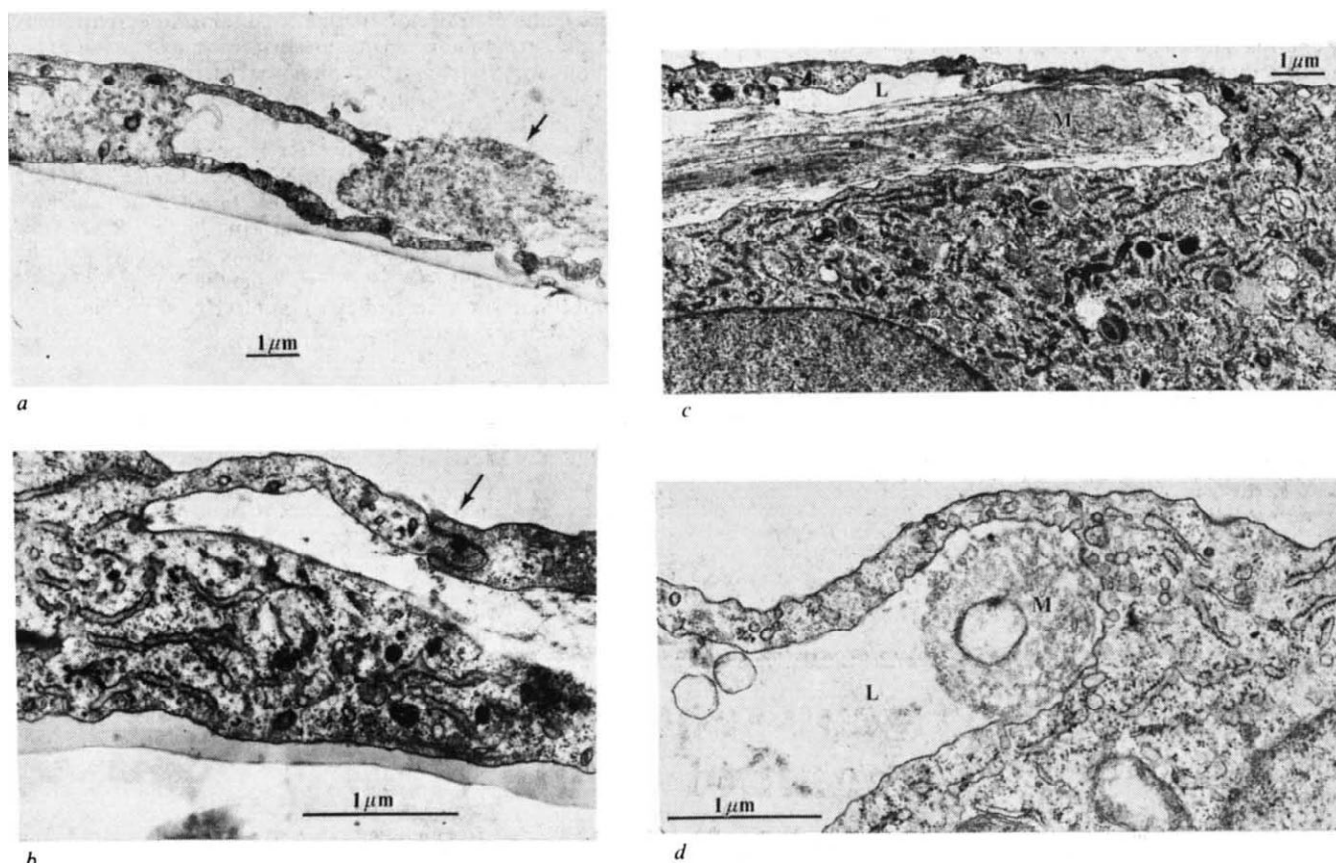


Fig. 5 Transmission electron micrographs of capillary formation. *a*, Section (perpendicular to the plane of the culture dish) of a human capillary endothelial cell showing two cytoplasmic extensions at the open end of a tube partially filled with extracellular material (arrow). *b*, Human capillary endothelial cell (cross-section perpendicular to the plane of the dish). A cytoplasmic extension forms a connection resembling a junctional complex (arrow) with another cytoplasmic extension from the same cell. *c*, Micrograph taken parallel to the plane of the culture dish, showing lumen (L) beginning to form within one capillary endothelial cell derived from human foreskin. Note the fibrillar nature of the intraluminal material (M) appearing like a 'mandril' within the lumen. N, nucleus. *d*, Lumen formation by human capillary cells derived from an ovarian tumour. Note the increased number of pinocytotic vesicles in the vicinity of the sparse material (M) remaining in the lumen (L).

formation by other cells, the following non-capillary cells were cultured in the same conditions that caused tube formation by human and bovine capillary endothelial cells—human foreskin fibroblasts obtained during the isolation of endothelial cells from foreskin were cultured for 4 months. Primary cultures of human kidney epithelium and umbilical vein endothelium were maintained for 4 months and human chondrocytes for 2 months. Also, WI-38 cells, BALB/c 3T3 fibroblasts, MDCK canine kidney cells and bovine aortic endothelial cells were cultured for up to 4 months. No tubes formed in any of these cultures.

Discussion

Our experiments show that all the information necessary to construct a 'capillary' tube, make branches and assemble an entire 'capillary' network *in vitro* can be expressed by a single cell type, the capillary endothelial cell. Other cell types are unnecessary. Therefore, mast cells and pericytes so frequently associated with capillaries *in vivo*, may have functions other than the formation and remodelling of the lumen. The unexpected finding is that cultured cells can be induced to mould into a tissue by themselves building a complex three-dimensional matrix. A further implication is that blood flow and hydrostatic pressure are not required for the organization of a vascular bed.

A general mechanism of microvascular lumen formation is suggested by the formation of an autolytic vacuole within a capillary endothelial cell, the conversion of this vacuole to a tube, the connection of one tube to another, the girdling of the tube by capillary endothelial cells that form special junctions with themselves and with each other and the ability of these cells to glide along the tube once it has formed. If these *in vitro*

observations are a valid model for growing capillaries *in vivo*, our understanding of how capillaries regenerate in wounds, proliferate at the edge of a tumour or arise from blood islands in the embryo may be enhanced.

We do not know why capillaries take so long to appear in primary cultures. One possibility is that gelatin is not the optimum substratum and the cultured capillary endothelial cells must lay down their own matrix^{12,13}. In addition, selection may be occurring, because capillary tubes arise earlier after passage of the endothelial cells. It is highly probable that the culture medium is not optimal. The medium used in this study was found empirically after several years of failure to grow capillary endothelium. Once we observed the first tube formation, the composition of the medium was not varied. The nature of the contents of the early vacuoles, the fibrils within the 'mandril', and the mechanisms of the removal of the material from within the tubes are unknown.

These experiments do not imply that only capillary endothelial cells are the source of new capillaries. We have limited our primary cultures to capillary segments. It is well known, however, that *in vivo* new capillaries arise from venules as well as from other capillaries.

This is the first report of angiogenesis *in vitro*. Because a variety of angiogenesis factors have been reported, based on *in vivo* assays^{8,14-20}, it will become critical to be able to distinguish between an indirect and a direct angiogenesis factor. By indirect, we mean compounds (for example formic acid and silica) that have in common the ability to attract macrophages or other white cells which might subsequently secrete a direct angiogenesis factor^{18,19}. An indirect angiogenesis factor would be

unable to induce capillary formation *in vitro*, where only capillary endothelial cells were present. By contrast, a direct angiogenesis factor should be capable of inducing angiogenesis *in vivo* or *in vitro* independently of other cell types. As yet there is no way to make this distinction. In the future, capillary endothelial cell cultures may be used to discriminate between indirect and direct angiogenesis factors. Furthermore, this approach may be used to screen for angiogenesis inhibitors.

A better understanding of angiogenesis in general, and tumour angiogenesis in particular, will be achieved if the various components of this phenomenon can be studied *in vitro*. At least one new concept is immediately apparent. If capillary endothelial cells have the capacity to generate a whole capillary

network (in the presence of tumour-conditioned medium) then the function of a tumour angiogenesis factor may be to supply instructions for 'start' and for 'direction' of capillary growth.

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- Gimbrone, M. A. Jr, Leapman, S. B., Cotran, R. S. & Folkman, J. *J. exp. Med.* **136**, 261–276 (1972).
- Folkman, J. *Ann. intern. Med.* **82**, 96–100 (1975).
- Folkman, J. & Cotran, R. S. *Int. Rev. exp. Path.* **16**, 207–248 (1976).
- Ausprunk, D. H. & Folkman, J. *Microvasc. Res.* **14**, 53–65 (1977).
- Ausprunk, D. H., Faltermann, K. & Folkman, J. *Lab. Invest.* **38**, 284–294 (1978).
- Folkman, J., Merler, E., Abernathy, C. & Williams, G. *J. exp. Med.* **133**, 275–288 (1971).
- Gimbrone, M. A. Jr, Cotran, R. S., Leapman, S. B. & Folkman, J. *J. natn. Cancer Inst.* **52**, 413–427 (1974).
- Klagsbrun, M., Knighton, D. & Folkman, J. *Cancer Res.* **36**, 110–114 (1976).
- Folkman, J., Haudenschild, C. C. & Zetter, B. R. *Proc. natn. Acad. Sci. U.S.A.* **76**, 5217–5221 (1979).

- Zetter, B. R. *Nature* **285**, 41–43 (1980).
- Gajdusek, C., Dicorlet, P. & Ross, R. *J. Cell. Biol.* **85**, 467–472 (1980).
- Howard, B. V., Macarak, E. J., Gunson, D. & Kefalides, N. A. *Proc. natn. Acad. Sci. U.S.A.* **73**, 2361–2364 (1976).
- Birdwell, C. R., Gospodarowicz, D. & Nicolson, G. L. *Proc. natn. Acad. Sci. U.S.A.* **7**, 3273–3277 (1978).
- Brown, R. A., Weiss, J. B. & Tomlinson, I. W. *Lancet* **i**, 682–685 (1980).
- Fenselau, A. & Mello, R. *J. Cancer Res.* **36**, 3269–3273 (1976).
- Fenselau, A. *et al. Fedn Proc.* **39**, 1615 (1980).
- Ryan, T. J. *Brit. J. Derm.* **82**, Suppl. 5, 99–111 (1970).
- Polverini, P. J., Cotran, R. S., Gimbrone, M. A. Jr & Unanue, E. R. *Nature* **269**, 804–806 (1977).
- Auerbach, R. & Sidky, Y. A. *J. Immun.* **123**, 751–754 (1979).
- Schor, A. M., Schor, S. L. & Kumar, S. *Br. J. Cancer* **40**, 302 (1978).

Chloroplast gene sequence for the large subunit of ribulose biphosphatecarboxylase of maize

Lee McIntosh, Carsten Poulsen & Lawrence Bogorad

The Biological Laboratories, Harvard University, Cambridge, Massachusetts 02138

The chloroplast gene coding for the large subunit of ribulose 1,5-bisphosphatecarboxylase in Zea mays has been sequenced. It contains neither a 'classical' prokaryotic promoter sequence nor its proposed eukaryotic counterpart but does have a typical prokaryotic ribosome binding site close to the site at which translation is initiated. Almost all possible codons occur and are translated by the 'universal code' rather than by the variation of it found in mitochondria.

THE enzyme ribulose biphosphatecarboxylase (RuBPCase) fixes CO₂ in plants and is the most abundant soluble leaf protein; it is also thought to be the most abundant protein on earth¹. It is an aggregate of eight identical 12,000–14,000 molecular weight (MW) 'small' subunits together with eight identical 'large' subunits (LS) of 50,000–55,000 MW (ref. 2). The small subunit of RuBPCase is inherited biparentally in *Nicotiana* and thus appears to be coded for by a nuclear gene³ which, in peas, is found among the population of unique or low copy sequences of the nuclear genome⁴. The large subunit, however, is encoded by chloroplast DNA for not only is it inherited maternally in *Nicotiana*⁵ but it can also be physically mapped on chloroplast DNA in *Zea mays*^{6–8} and in *Chlamydomonas reinhardtii*⁹. This distribution of the genes for the two subunits of RuBPCase between the nuclear and chloroplast genomes is an example of the widespread phenomenon of the dispersal of genes for the components of multimeric proteins of subcellular organelles¹⁰.

We report here the complete DNA sequence of the gene for the LS of *Z. mays* RuBPCase contained in a fragment of the chloroplast genome that has been cloned in *Escherichia coli*^{6–8} and mapped on the cloned fragment^{7,8}. The transcribed portion of the DNA sequence has some features in common with genes of other groups of organisms but overall is unique. The codons used for translation of LS mRNA include most of those possible, which has led us to consider the kinds of tRNA genes that may be found in the maize chloroplast chromosome. Furthermore, the amino acid sequence deduced from the DNA sequence has been compared and aligned with amino acid sequences obtained

earlier for some cyanogen bromide (CNBr) fragments of barley LS¹¹ and with sequences of tryptic peptides of the spinach enzyme, believed to be in or near the catalytic sites¹². Stretches of the deduced amino acid sequence of maize LS are remarkably similar to the known amino acid sequences in the barley and spinach polypeptides, with the putative catalytic sites widely separated along the polypeptide.

Features of DNA sequence for LS RuBPCase mRNA

A physical map of the maize chloroplast DNA fragment that includes the structural gene for the LS of RuBPCase is shown in Fig. 1 together with the strategy used for sequencing the region complementary to the LS mRNA. The nucleotide sequence is shown in Fig. 2.

The 5' terminus of LS mRNA was previously estimated to be complementary to the DNA for about 40 nucleotides upstream from a mapped *EcoRI* restriction site⁸ (nucleotide –34 to –29 in Fig. 2). We have now placed the terminus more precisely using a modification¹³ of the Berk–Sharp technique¹⁴. The 5' terminus of the transcript was previously mapped within a 196-base pair *EcoRI* fragment that is part of a larger 480-base pair *HinfI* fragment (Fig. 1). This fragment was isolated and labelled with ³²P at its 5' ends by polynucleotide kinase¹⁵ and then digested with *HincII* endonuclease. The resulting radioactive 443-base pair *HinfI/HincII* fragment was purified by polyacrylamide gel electrophoresis and used for DNA:RNA hybridization and DNA sequencing (Fig. 3).

The radioactive segment recovered from the hybrid should represent the DNA protected from S_1 -nuclease digestion by hybridization with LS mRNA. Determination of its length should establish the precise beginning of the LS transcript. Protected fragments of 219, 220, 224 and 225 bases were detected as a set of two bands. This places the 5' end of the LS mRNA 59 to 63 nucleotides from the start of the codon for the N-terminal methionine residue (nucleotide 1 in Fig. 2). Removal of a 3'-terminal phosphate by the S_1 nuclease increases the electrophoretic mobility of the fragment by about 1/2 base in relation to the sequence ladder¹³.

Five nucleotides upstream from the N-terminal methionine codon on the LS mRNA is the sequence 5'-GGAGG-3' that is complementary to a sequence at the 3' terminus of maize chloroplast 16S rRNA¹⁶. A sequence identical with the latter is found at the 3' terminus of 16S rRNA in *E. coli* and a complementary sequence is generally found on bacterial mRNAs, about three to seven nucleotides upstream from the initiator codon. The suggestion by Shine and Dalgarno¹⁷ that this complementary mRNA sequence is a translation signal was later confirmed by identification of hybrids between mRNA and sequences at the 3' terminus of 16S rRNA¹⁸. The entire 'Shine and Dalgarno' nine-nucleotide sequence of *E. coli* 16S rRNA is present in maize chloroplast 16S rRNA but only seven of the nine nucleotides at positions 5-13 upstream from the initiator codon on the LS mRNA are complementary to the maize chloroplast 16S rRNA Shine-Dalgarno sequence (Fig. 2). Another partially complementary sequence has been detected on a different maize chloroplast DNA fragment upstream of a putative translation start site for an unidentified polypeptide. (Z. Schwarz, A. Steinmetz and L. Bogorad, unpublished). Partially complementary sequences have also been seen in the *cro* gene (5'-A-A-G-G-A-G-G-3'), the *O* gene (5'-A-G-G-A-G-3') and in the *CII* gene (5'-A-A-G-G-A-3') of λ phage¹⁹.

Amino acid sequence of maize LS RuBPCase

The amino acid sequence deduced from the nucleotide sequence of the structural region of the LS gene is shown in Fig. 2. The polypeptide comprises 475 amino acid residues and has a molecular weight of 52,682. This number of residues is lower than previously estimated from electrophoretic and amino acid analyses of LS of RuBPCase from species other than maize²⁰.

The availability of the DNA sequence for the maize LS RuBPCase gene has permitted the alignment of amino acid sequences totalling 244 residues known for fragments of the barley and spinach LS polypeptides^{11,12} with the predicted maize LS sequence (Fig. 2). There are few differences. Seven positions in the maize sequences have amino acid replacements due to single nucleotide substitutions and five of these (positions 36,

43, 282, 394 and 460) must be considered neutral replacements. The proline residues at positions 46 and 415 in the barley LS primary structure are replaced by leucine and histidine respectively. The application of methods for secondary-structure prediction²¹ to these domains in the sequences suggest that they are not likely to have any drastic effect on secondary structure. The eighth difference, at amino acid residue 215, may be the result of an error in identification of that residue in the barley polypeptide.

Compared with the barley polypeptide²⁰, the maize LS mRNA seems to contain a sequence for 14 amino acid residues that extends beyond the N-terminus of the barley LS sequence. This difference could be due to variation in the DNA sequences, but it seems more likely that the difference is an artefact. After SDS polyacrylamide gel electrophoresis of fragments produced by CNBr-cleavage of the large subunits from maize and from rapidly isolated barley RuBPCase, we found N-terminal fragments of identical size (MW 14,000-15,000, data not shown). The N-terminal alanine, which has previously been reported for the LS of barley¹¹, was not found among the CNBr-fragments of either maize or barley. The N-terminal amino acid could not be identified for intact maize or barley LS polypeptides, which suggests that these are blocked, possibly by N-formyl-methionine. N-terminal blocking has now been observed for large subunits from a number of species¹¹. It is possible that the 14 N-terminal residues of the barley LS are removed by an endo-protease present in the barley leaf extracts during the slower large-scale preparations of the enzyme¹¹.

The amino acid sequences of spinach LS that have been proposed to be in or near the catalytic site of RuBPCase¹² are widely separated along the maize polypeptide, corresponding to positions 166-178, 321-340 and 450-462, and are well conserved. The only exceptions are neutral replacements at positions 330 and 460. Note that nine histidines are clustered among residues 268-327 and that this is just on the N-terminal side of one of the proposed catalytic site sequences. Four of these histidines are found in two His-Ile-His sequences (292-294 and 325-327). Whether any of the clustered imidazole groups are used for chelation of Mg^{2+} , which is required for enzyme activity, is not known. Note also that many lysine, arginine and cysteine residues are in the middle of the sequence and close to the C-terminus. Some side chains of these residues are thought to have important roles in binding to, or acting on, the substrates^{12,21,22}. The positions in which they have been found might be close to the three proposed catalytic site sequences.

The complete amino acid sequence deduced for the maize LS polypeptide clarifies some ambiguities in previously published data²⁰ on the amino acid sequence of the barley LS: (1) An

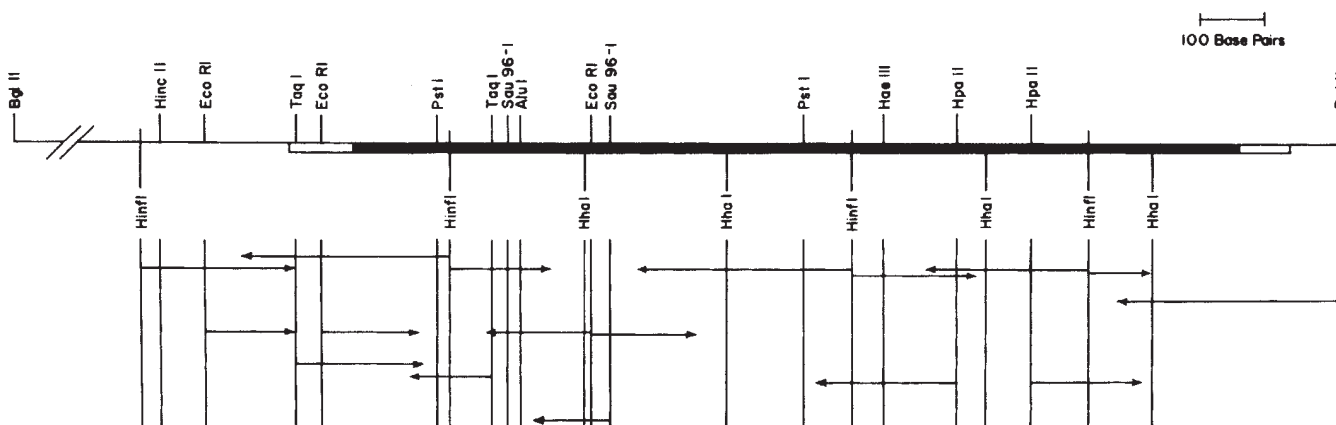
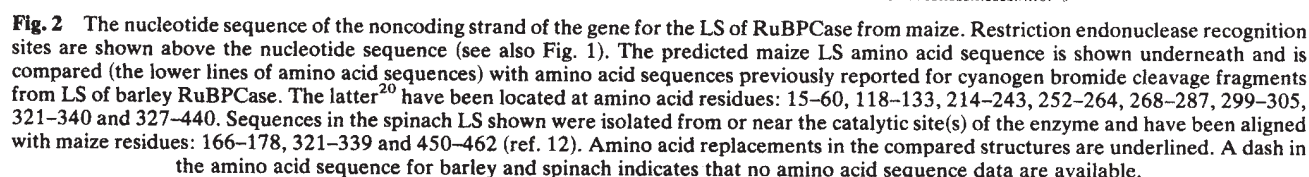


Fig. 1 Fine restriction endonuclease map and the strategy followed for sequencing the maize chloroplast DNA sequence containing the gene for LS RuBPCase. The recombinant plasmid pZmc 3711 is comprised of a 2.7-kilobase pair maize chloroplast DNA fragment bearing the LS gene⁷ incorporated into resistance plasmid RSF 1030 (ref. 8). The double-lined region indicates the boundaries of the mature LS transcript and the solid rectangle within represents the amino acid coding part of the sequence. The sequencing strategy is indicated by the arrows which originate from specific endonuclease restriction sites that were labelled at their 5' ends with [γ -³²P]ATP. DNA sequence determination, end labelling and electrophoresis were according to the procedures of Maxam and Gilbert¹⁵.



amino acid sequence corresponding to residues 118–113 has previously been reported as arising from the barley small subunit²³ but the absence of a similar sequence from the spinach small subunit²⁴ and the presence of this sequence in the maize LS primary structure indicate that it was a contaminant from the large subunit. (2) An undecapeptide corresponding to amino acid positions 310–320 of maize LS was not detected on isolation of CNBr-fragments from the barley LS although the two surrounding CNBr fragments were found²⁰. Failure to identify this undecapeptide in the course of the work on the barley enzyme might have confused the conclusions drawn from the sequencing of the peptide fragment 298–309. Although differences at one or two positions cannot be excluded, it seems likely that the two sequences are similar in this region. (3) The size of the fragment 252–267 was earlier over-estimated²⁰ due to abnormal chromatographic behaviour and the presence of a larger contaminating peptide. (4) The extra tryptophan thought to be present in the sequence 368–383 of the LS in barley²⁰ seems to be absent. (5) The assignment of isoleucine instead of tryptophan to a position in the barley LS corresponding to residue 215 in the maize LS does not comply with the simple nucleotide substitution patterns observed for the eight replacements described above. However, phenylthiohydantoin derivatives of isoleucine and tryptophan may have been confused in the HPLC system used for their identification²³. Tryptophan has been detected in the sequence 214–243 in a Trp-Arg peptide after tryptic digestion of the corresponding barley CNBr fragment²⁰. Because no other tryptophans in this sequence can be deduced from the nucleotide sequence, tryptophan is the most likely residue at this position in the barley LS.

The major difference in the amino acid sequence seems to be that between the C-termini of the barley and maize large subunits—the barley LS showed Leu-Ala-Val-COOH as C-terminal residues. This sequence cannot be obtained from the maize LS C-terminus by nucleotide sequencing. It is possible that this discrepancy is an artefact of the same type as described for the N-terminus and further study of the barley LS gene may clarify this.

Codon usage in the LS gene

Figure 4 shows the frequency with which amino acid codons are used in the synthesis of the LS of maize RuBPCase. All 20 amino acids are used and all codons except for three which may be used in other chloroplast genes. There is a strong bias towards having adenine and thymine bases in the third position of the codons used. In the maize LS gene 178 thymine, 145 adenine, 78 cytosine and 75 guanine bases occur in the third position of the codon. These distributions are similar to those found for codon usage in the human mitochondrial gene for cytochrome oxidase subunit II²⁵. However, we did not find the stop codon, UGA, used for tryptophan as it is in the mRNA for cytochrome oxidase II from human²⁵ and yeast mitochondria²⁶.

The use of almost all codons possible in the LS mRNA seems to be inconsistent with earlier results for the number of tRNA genes in maize chloroplasts. Saturation-hybridization studies conducted with maize cpDNA and chloroplast tRNAs gave an estimate of 20–26 tRNA genes on the maize chloroplast chromosome²⁷. Similar types of hybridization experiments have given estimates of 18–25 chloroplast tRNA genes for *Euglena*²⁸ and 30–40 tRNA genes for pea²⁹.

Recently these hybridization data have been complemented by separation of chloroplast 4S RNAs in two-dimensional gel electrophoresis and identification of the individual tRNAs by charging them using *E. coli* and chloroplast aminoacyl tRNA synthetases^{30,31}. Maize chloroplast tRNAs separated in this manner yielded approximately 28 spots of which 25 were charged by 17 amino acids.

If, as predicted by the LS gene, almost all codons were used, a minimum of 32 tRNAs are required if the G–U wobble hypothesis is followed³². This is inconsistent with the saturation-hybridization data²⁷ but the conditions in those experiments may not have favoured hybridization of all the tRNAs. In

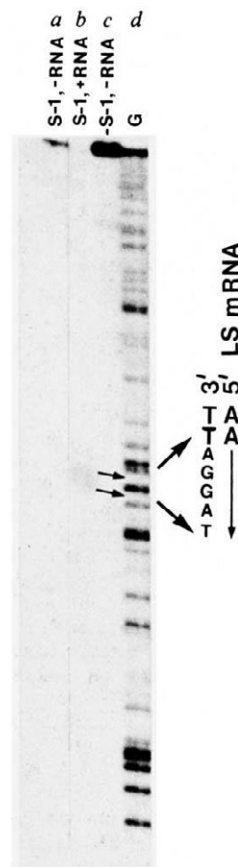


Fig. 3 Localization of the 5' terminus of the LS mRNA on the coding strand of the LS gene. This experiment was carried out using a modification of the method of Berk and Sharp¹⁴. A ³²P 5'-terminally labelled DNA fragment was denatured and hybridized with RNA samples, digested with S₁ nuclease, and the length of the protected fragment was measured by polyacrylamide gel electrophoresis in 8.3 M urea. Total maize chloroplast RNA was obtained from isolated chloroplasts⁸. The DNA probe was a 443 base pair *HinfI*/*HincII* DNA fragment (see Fig. 1) with the 5' end labelled at the *HinfI* restriction endonuclease site, that is, on the coding strand of DNA for the LS gene. The *HinfI*/*HincII* fragment was denatured at 65 °C in flame-sealed 10-μl siliconized glass capillary pipettes. The 10-μl hybridization mixture was composed of 80% w/v deionized formamide, 0.4 M NaCl, 0.04 M PIPES (pH 6.4), 1 mM EDTA, 20–50 ng DNA probe and 30 μg of total chloroplast RNA^{8,14}. Digestion was carried out with 20 units of S₁ nuclease in a total volume of 110 μl of dilution buffer (0.25 M NaCl, 0.02 M NaOAc, 1 mM ZnSO₄, 20 μg ml⁻¹ freshly denatured calf thymus DNA) for 20 min at 40 °C. The S₁-resistant material was precipitated with ethanol and resuspended in 80% formamide, 50 mM Tris-borate (pH 8.3), 1 mM EDTA, and electrophoresed on 6% polyacrylamide 8.3 M urea gels. Results are shown in the autoradiograph. The sample in lane a was probe DNA treated with S₁ nuclease in the absence of chloroplast RNA—the only DNA band seen is a residual amount of double-stranded (therefore S₁ nuclease resistant) probe. The DNA used in lane c was subjected to hybridization incubation without RNA. It was not treated with S₁ nuclease. The DNA in lane b was from the reaction mixture in which RNA was present during hybridization incubation and treatment. Two wide bands are seen representing four DNA fragments protected from digestion by hybridization with LS mRNA. Their sizes and positions in the DNA sequence were determined by comparison with the G-reaction for the original DNA fragment, shown in lane d.

		Second letter							
First letter		U		C		A		G	
U	Phe	12	Ser	6	Tyr	9	Cys	6	U
	Phe	9	Ser	4	Tyr	8	Cys	5	C
	Leu	11	Ser	5	ochre	1	opal	0	A
	Leu	12	Ser	1	amber	0	Trp	8	G
C	Leu	5	Pro	6	His	11	Arg	11	U
	Leu	1	Pro	2	His	4	Arg	9	C
	Leu	7	Pro	9	Gln	11	Arg	3	A
	Leu	1	Pro	4	Gln	2	Arg	0	G
A	Ile	11	Thr	17	Asn	6	Ser	3	U
	Ile	9	Thr	7	Asn	7	Ser	0	C
	Ile	4	Thr	6	Lys	18	Arg	4	A
	Met	1 + 11	Thr	0	Lys	8	Arg	2	G
G	Val	12	Ala	18	Asp	24	Gly	21	U
	Val	1	Ala	6	Asp	6	Gly	1	C
	Val	14	Ala	18	Glu	20	Gly	13	A
	Val	3	Ala	5	Glu	8	Gly	9	G

Fig. 4 Use of codons in the translation of the maize LS mRNA. All codons but three are used. The three codons not used, ACG(Thr), CGG (Arg) and AGC (Ser), may be used for other chloroplast proteins.

addition, too few tRNAs are revealed by two-dimensional separation of chargeable tRNAs³¹. It is possible that specific tRNAs are preferentially lost and/or degraded during isolation, that some may be present in amounts below the level of detection, or that chloroplast tRNAs may not all be chargeable with the *E. coli* tRNA aminoacyl synthetases usually used³¹. Another possibility is that tRNAs are superimposed on one another in two-dimensional electrophoresis. The latter has occurred in one case, where a tRNA^{Leu} spot contains two isoacceptors (A. Steinmetz, personal communication).

There is no firm evidence that RNA is transported into higher-plant chloroplasts. It may therefore be reasonable to assume that chloroplasts contain a full complement of tRNAs, some of which have not been detected. However, it is possible that chloroplasts of some species contain only a limited set of tRNAs and use a different protein synthesizing system, or an alternative codon reading method such as the 'two out of three' system³³ could be used. This mechanism requires that only the first two bases be read in codon recognition and thus only 24 tRNAs be required.

Conclusions

A number of features of transcribed and adjacent non-transcribed portions of the maize gene for LS of RuBPCase can be enumerated: (1) The two ribonucleotides at the 5' terminus of LS mRNA are most probably adenosines (AA, see Fig. 3)—other experiments (S. O. Jolly, L. McIntosh and L. Bogorad, unpublished) show that maize chloroplast DNA-dependent RNA polymerase initiates transcription *in vitro* at a position equivalent to the 5' end of the LS mRNA in the presence of the S factor described by Jolly and Bogorad³⁴. Thus, the 5'-terminus

of LS mRNA is very likely to be at the site of initiation of transcription and is not processed. (2) The mRNA transcript carried an almost complete Shine-Dalgarno sequence a few nucleotides upstream from the translation initiation codon. Preliminary evidence (Z. Schwarz, A. Steinmetz and L. Bogorad, unpublished) indicates that at least one other mRNA in the chloroplast may carry this type of prokaryotic signal sequence. (3) It is not known whether chloroplast mRNAs are capped, as are eukaryotic and viral mRNAs³⁵, but the LS DNA sequence shows no apparent homology to identified capping sequences^{36,37}. Thus, unless some entirely different signal sequence is recognized by a chloroplast capping enzyme, LS mRNA is similar to prokaryotic messages in lacking a cap. (4) No DNA sequence homologous to a classical bacterial promoter³⁸ nor its presumed eukaryotic counterpart, the 'Hogness' box³⁹, is found upstream from the transcribed portion of the maize LS gene. (5) The colinearity of LS mRNA with the DNA sequence is confirmed. (6) Almost all possible codons are used in the LS gene. (7) Analysis of the codon usage by the LS mRNA indicates that, unlike fungal²⁶ and mammalian²⁵ mitochondria, the universal genetic code is used in chloroplasts.

It is not known how many of the features listed will prove to be universal for chloroplast mRNAs although it would not be surprising if many were general. Another characteristic which may be universal is the striking conservation of amino acid sequences between the LS polypeptides of maize, barley and spinach. Only seven or eight amino acid substitutions have been detected among 228 (of 475) amino acids sequenced for LS in barley²⁰ and corresponding portions of the deduced amino acid sequence in maize. It is possible that sequence conservation of multimeric enzymes, especially those whose components are dispersed in two different genomes¹⁰, will prove to be common.

In understanding the three-dimensional structure of this polypeptide and the mechanism of its enzymatic action, it is interesting that the active site sequences are widely separated in the maize polypeptide; thus, extensive folding must be required to bring these sequences close together.

The hypothesis that chloroplasts and mitochondria evolved from prokaryotic symbionts in pro-eukaryotic cells has been well received. Evidence to support this view has been mainly the presence of 'prokaryotic features' in organelle nucleic acids and proteins and the 'retention' of presumed biochemical relics. The alternative genetic code used by mitochondria, that is, UGA coding for tryptophan^{25,26}, and the failure to detect prokaryotic-type promoter sequences adjacent to chloroplast genes emphasize that organelles—regardless of their origin—are unique.

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- Kung, S.-D. *Science* **191**, 429–434 (1976).
- Kawashima, N. & Wildman, S. G. *A. Rev. Pl. Physiol.* **21**, 325–358 (1970).
- Kawashima, N. & Wildman, S. G. *Biochim. biophys. Acta* **262**, 42–49 (1972).
- Cashmore, A. R. *Cell* **17**, 383–388 (1979).
- Chan, P. H. & Wildman, S. G. *Biochim. biophys. Acta* **277**, 677–680 (1972).
- Coen, D. M., Bedbrook, J. R., Bogorad, L. & Rich, A. *Proc. natn. Acad. Sci. U.S.A.* **74**, 5487–5491 (1977).
- Bedbrook, J. R., Coen, D. M., Beaton, A. R., Bogorad, L. & Rich, A. *J. biol. Chem.* **254**, 905–910 (1979).
- Link, G. & Bogorad, L. *Proc. natn. Acad. Sci. U.S.A.* **77**, 1832–1836 (1980).
- Malnoe, P., Rochaix, J.-D., Chua, N. H. & Spahr, P.-F. *J. molec. Biol.* **133**, 417–434 (1979).
- Bogorad, L. *Science* **188**, 891–898 (1975).
- Poulsen, C., Martin, B. & Svendsen, I. *Carlsberg Res. Commun.* **44**, 191–199 (1979).
- Hartman, F. C., Norton, I. L., Stringer, C. D. & Schloss, J. V. in *Photosynthetic Carbon Assimilation* (eds Siegelman, H. W. & Hind, G.) 245–269 (Plenum, New York, 1978).
- Moss, T. & Birnstein, M. L. *Nucleic Acids Res.* **7**, 3733–3743 (1979).
- Berk, A. J. & Sharp, P. A. *Cell* **11**, 721–732 (1977).
- Maxam, A. & Gilbert, W. *Proc. natn. Acad. Sci. U.S.A.* **74**, 560–564 (1977).
- Schwarz, Zs. & Kossel, H. *Nature* **279**, 520–523 (1979).
- Shine, J. & Dalgarno, L. *Proc. natn. Acad. Sci. U.S.A.* **71**, 1342–1346 (1974).
- Steitz, J. A. & Jakes, K. *Proc. natn. Acad. Sci. U.S.A.* **72**, 4734–4738 (1975).
- Schwartz, E., Scherer, G., Hobom, G. & Kossel, H. *Nature* **272**, 410–414 (1978).
- Poulsen, C. *Carlsberg Res. Commun.* **44**, 163–189 (1979).

- Paech, C., McCurry, S. D., Pierce, J. & Tolbert, N. E. in *Photosynthetic Carbon Assimilation* (eds Siegelman, H. W. & Hind, G.) 227–243 (Plenum, New York, 1978).
- Sugiyama, T., Nakayama, N., Ogawa, M., Akazawa, T. & Oda, T. *Archs Biochem. Biophys.* **125**, 98–106 (1968).
- Poulsen, C., Strobeck, S. & Haslett, B. G. in *Genetics and Biogenesis of Chloroplasts and Mitochondria* (eds Bucher, Th., Neupert, W., Sebald, W. & Werner, S.) 17–24 (1976).
- Martin, P. G. *Aust. J. Pl. Physiol.* **6**, 401–408 (1979).
- Barrell, B. G., Bankier, A. T. & Drouin, J. *Nature* **282**, 180–194 (1979).
- Fox, T. D. *Proc. natn. Acad. Sci. U.S.A.* **76**, 6534–6538 (1979).
- Haff, L. A. & Bogorad, L. *Biochemistry* **15**, 5105–5109 (1976).
- McCreary, J. M. & Hersberger, C. L. *Nucleic Acids Res.* **3**, 2005–2018 (1976).
- Tewari, K. K., Kolodner, R., Chu, N. M. & Meeker, R. R. in *Nucleic Acids and Protein Synthesis in Plants* (eds Bogorad, L. & Weil, J.-H.) 15–36 (Plenum, New York, 1977).
- Driessell, A. J. *et al. Gene* **6**, 285–306 (1979).
- Mubumbila, M. *et al. Biochim. biophys. Acta* (in the press).
- Crick, F. H. J. *molec. Biol.* **19**, 548–555 (1966).
- Lagerkvist, U. *Proc. natn. Acad. Sci. U.S.A.* **75**, 1759–1762 (1978).
- Jolly, S. O. & Bogorad, L. *Proc. natn. Acad. Sci. U.S.A.* **77**, 822–826 (1980).
- Shatkin, A. J. *Cell* **9**, 645–653 (1976).
- Lai, E. C. *et al. Cell* **18**, 829–842 (1979).
- Ziff, E. B. & Evans, R. M. *Cell* **15**, 1463–1474 (1978).
- Pribnow, D. *Proc. natn. Acad. Sci. U.S.A.* **72**, 784–788 (1975).
- Busslinger, M., Portmann, Irminger, T. C. & Birnstein, M. L. *Nucleic Acids Res.* **8**, 957–977 (1980).

Recognition sequence of bacteriophage Φ X174 gene A protein—an initiator of DNA replication

A. D. M. van Mansfeld^{*†}, S. A. Langeveld^{*}, P. D. Baas^{*†}, H. S. Jansz^{*†},
G. A. van der Marel[‡], G. H. Veeneman[‡] & J. H. van Boom[‡]

^{*} Institute of Molecular Biology and [†] Laboratory for Physiological Chemistry, State University of Utrecht, Utrecht, The Netherlands

[‡] Department of Organic Chemistry, State University of Leiden, Leiden, The Netherlands

Gene A protein, the initiator protein of bacteriophage Φ X174 DNA replication, cleaves synthetic single-stranded oligodeoxyribonucleotides at the same site as the corresponding sequence at the Φ X origin. The results identify the recognition sequence within the decamer CAACTTGATA which is cleaved next to the G residue. Further requirements for cleavage of double-stranded DNA by the gene A protein are supercoiling and an A + T-rich domain adjacent to the recognition sequence.

THE single-stranded circular Φ X DNA replicates via a double-stranded circular intermediate, the replicative form (RF)¹. Φ X DNA replication is initiated by the introduction of a single nick in one strand, the viral strand, of the RF DNA by the virus-coded gene A protein². *In vivo*³ and *in vitro* studies⁴⁻⁶ have shown that this nick resides at a unique site of the Φ X RF DNA molecule and that Φ X DNA replication starts at this nick. Therefore, the site of the gene A protein nick in Φ X RF DNA is the origin of DNA replication. Because only two macromolecular components—the DNA and the gene A protein—are involved, the Φ X system is particularly favourable for studying the initiator–origin interaction at the molecular level.

Previous studies from our laboratory⁴ have shown that the purified gene A protein nicks the viral strand of Φ X RF DNA,

creating a 3'OH-G residue at position 4,305 of the Φ X DNA sequence⁷. After nicking, the gene A protein remains covalently bound at the 5'-end of the nick^{4,6,8}. Further studies have shown that the RFI DNAs of the bacteriophages G4, St-1, U3, G14 and α 3 are also nicked by the Φ X gene A protein⁹⁻¹¹. Comparison of the sequences around the nick sites in Φ X, G4 and St-1 shows that the three phages have an uninterrupted stretch of 10 nucleotides, CAACTTGATA, in common¹⁰. This suggests that the recognition site of the Φ X gene A protein lies within this 10-nucleotide sequence.

The present study is concerned with the further elucidation of the recognition nucleotide sequence of the Φ X gene A protein, and used synthetic oligodeoxyribonucleotides as substrates for the purified gene A protein. This approach was suggested by the

Table 1 Oligonucleotides obtained by partial digestion of CAACTTGATATTAATA with snake venom (a) and spleen (b) phosphodiesterase and incubation with or without gene A or A* protein

a Length	% Of total*		b Length	% Of total*		
	–Gene A protein	+Gene A protein		–Gene A or A* protein	+Gene A protein	+Gene A* protein
16	7.9	1.5	16	25.5	7.7	6.9
15	8.1	1.0	15	18.7	12.3	6.3
14	4.2	—	14	24.6	19.3	7.4
13	2.7	—	13§	21.3	15.4	15.2
12	1.7	—	12§	10.1	6.0	5.7
11	2.9	—	11	—	2.2	2.0
10	3.1	1.9†	10	—	3.0	5.7
9	5.2	5.3	9	— ¹	—	—
8	9.0	9.8	8	—	2.3	—
7	12.6	37.8	7	—	21.2	17.6
6	8.9	10.2	6	—	4.5	11.1
5	10.1	10.1	5	—	3.4	19.9
4	8.1	7.0	4	—	0.4	27
3	4.0	3.7	3	—	—	—
2	1.9	1.7				
1	9.7	7.2				
11†	—	2.6				

For the incubation with the A* protein values for a were not determined.

* 100% In parts a and b is the sum of the radioactivity present in bands 1–16 and 3–16, respectively.

† This material represents unknown minor component which is also seen in Fig. 2, lane b.

‡ This value is relatively high, because an unknown minor component which is also seen in Fig. 2, lane b, also migrates at this position.

§ Although the oligonucleotides with chain lengths 13 and 12 are also degraded by incubation with the gene A protein or the A* protein, there is no corresponding appearance of ³²P-labelled tetra- and trinucleotide cleavage products. The most likely explanation for this is that during incubation with spleen phosphodiesterase, some degradation from the 3' end of the hexadecamer also occurs. This yields oligonucleotides shorter than 16 residues, all of which give rise to a ³²P-labelled cleavage product seven residues long.

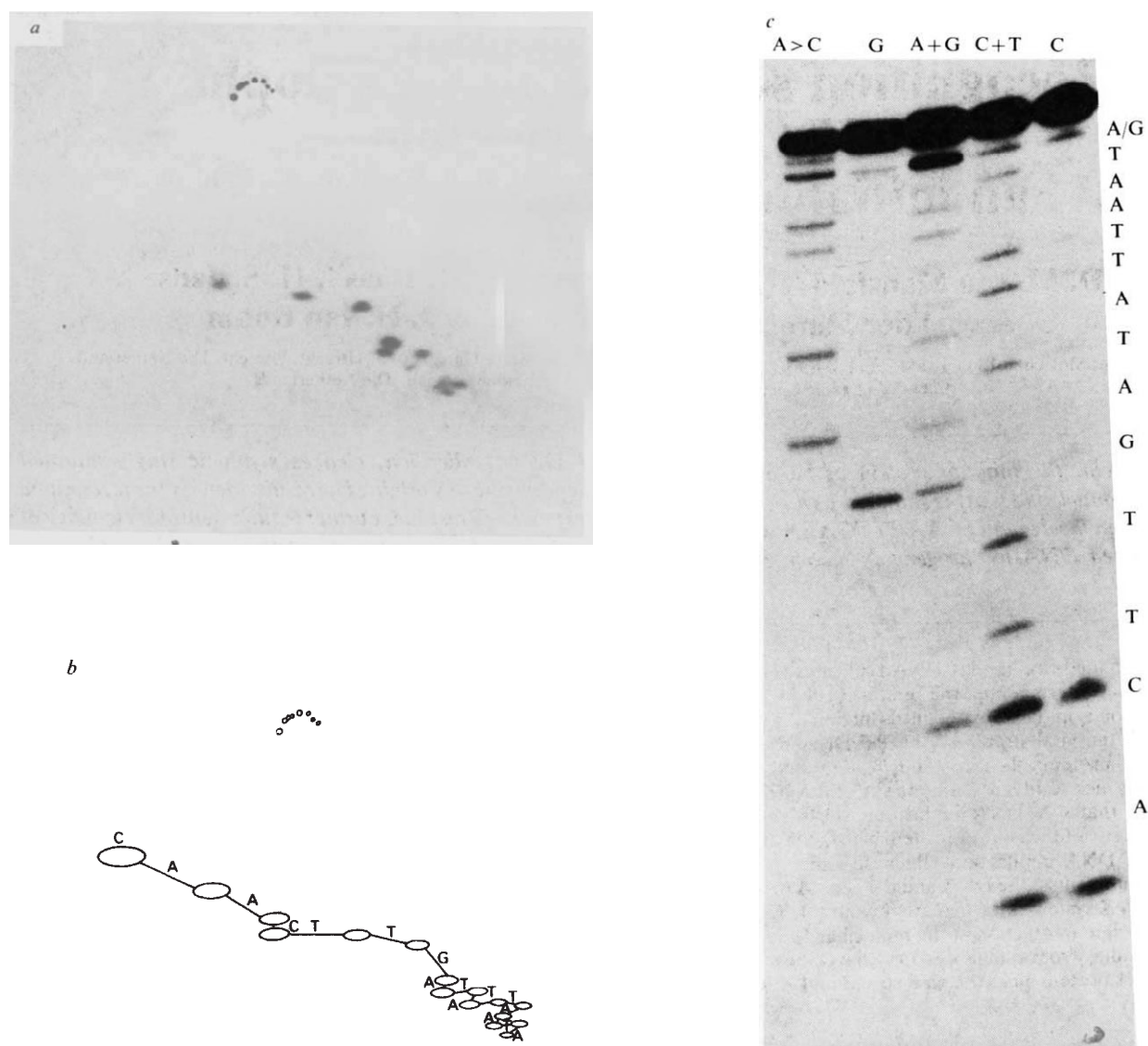


Fig. 1 Characterization of the synthetic hexadecamer. The synthetic hexadecamer was labelled at the 5' end with radioactive phosphate according to the protocol of Maxam and Gilbert¹⁹. [γ -³²P]ATP, $\sim 2,000$ Ci mmol⁻¹, was from the Radiochemical Centre and T4-induced polynucleotide kinase was from Boehringer Mannheim. The labelled oligonucleotide (0.18 pmol) was partially digested with snake venom phosphodiesterase (32 pg Worthington) by incubating for 1 or 20 min at 37 °C in 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 10 mM MgCl₂ and 5 mM dithiothreitol (DTT) in a volume of 20 μ l. The reaction was stopped by addition of 50 μ l phenol and vigorous mixing. After separating the phenol and water phase, the water phase was extracted with ether to remove residual phenol. The water phases of the different incubations (1 min and 20 min) were mixed. Aliquots (1.25 μ l) of this mixture were applied to a cellulose-acetate strip and the radioactive products were separated by electrophoresis (1st direction) and homochromatography (2nd direction) on a PEI-cellulose thin layer using a 3% 'homomix' and visualized by autoradiography as described before³⁵. The 5'-labelled oligonucleotide was also subjected to the chemical method for sequencing DNA devised by Maxam and Gilbert¹⁸, with modifications as described recently¹⁹. The digonucleotide was treated with alkali (the so-called A > C reaction), dimethylsulphate (G), piperidinium formate (A + G), hydrazine (C + T), and hydrazine in the presence of salt (C). To obtain a higher recovery of this short DNA fragment at the ethanol precipitations, the ethanol to water ratio was raised to 4:1 (instead of 3:1). After the modifications the cleavage reactions were performed with 1 M piperidine. The samples were then lyophilized repeatedly, dissolved in 7.5 μ l 98% formamide containing 0.05% bromophenol blue (BFB) and 0.05% xylene cyanol F (XFC) and applied onto a thin (0.45 mm) 25% polyacrylamide gel containing 50 mM Tris-borate (pH 8.35), 1 mM EDTA (TBE buffer) and 7 M urea. Electrophoresis was performed at 30 W until the XCF marker had migrated 9 cm. *a*, The autoradiograph of the radioactive products separated by electrophoresis and homochromatography. *b*, The interpretation of the autoradiograph. The capitals indicate which nucleotide causes the shift of an oligonucleotide to the next longer one. *c*, The autoradiograph of the sequencing gel, aligned with the sequence deduced from the successive bands.

finding that the gene A protein nicks single-stranded Φ X DNA only once and at exactly the same position as Φ X RFI DNA¹².

The gene A of bacteriophage Φ X not only codes for the gene A protein (molecular weight (MW) 55,000), but also for the A* protein (MW 37,000) which is formed from a natural internal initiator site within gene A¹³. The function of the A* protein is unknown. It does not nick Φ X RFI DNA. However, the purified A* protein can cleave the single-stranded viral DNA of Φ X at many different sites¹². Gene A protein and A* protein were purified as described recently¹⁴. Both proteins were available in pure form, and therefore we also tested the action of the A* protein on the synthetic oligonucleotides.

Characterization of the hexadecadeoxyribonucleotide

The hexadecamer CAACTTGATATTAATA was synthesized using the phosphotriester method^{15,16}. This sequence corresponds to nucleotides 4,299–4,314 in the Φ X DNA sequence⁷. It contains the gene A protein cleavage site (at the 3' side of the G residue) in Φ X DNA and the remarkable self-complementary sequences left (CAACTTG) and right (TATTAATA) of this site. It was thought that these self-complementary structures might have a role in the recognition between the gene A protein and the DNA.

Fig. 2 The nicking activity of the gene A protein and A* protein on the hexadecamer and derivatives. 5'-Labelled oligonucleotide (0.045 pmol) was incubated with gene A protein (0.9 pmol) or A* protein (0.72 pmol) in 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 10 mM MgCl₂, 5 mM DTT and 150 mM NaCl for 45 min at 37 °C in a volume of 12 µl. Then 1 µl 150 mM EDTA (pH 8.0) and 1 µl of a solution of proteinase K (Merck Darmstadt), 2 mg ml⁻¹ in 1 mM Tris-HCl (pH 7.5), 0.1 mM EDTA, preincubated for 30 min at 37 °C, were added and the incubation was continued for 30 min at 37 °C. Then 15 µl 98% formamide containing 0.05% XCF and 1 µl 1% sarkosyl (Ciba-Geigy) were added, the sample was heated for 30 s at 100 °C and applied to a 25% polyacrylamide gel in TBE buffer containing 7 M urea. Electrophoresis was carried out at 35 W until the XCF marker had migrated 9 cm. Autoradiography was performed at -20 °C. Instead of the hexadecamer, the mixture of oligonucleotides, obtained by shortening the hexadecamer from the 3' end by digestion with snake venom phosphodiesterase as described in Fig. 1 legend, was used. A third series of incubations was carried out with a mixture of oligonucleotides that had been obtained by shortening the hexadecamer from the 5' end; 150 pmol hexadecamer was incubated with 7.5 µg spleen phosphodiesterase (Boehringer Mannheim) in 20 µl 10 mM Tris-HCl (pH 7.5), 1 mM EDTA for 10 min at 37 °C. Phenol was added to stop the reaction. After vigorous mixing and separation of the phenol and water phase, the water phase was extracted with ether and the oligonucleotides were labelled at their 5' end as described in Fig. 1 legend. The oligonucleotide mixture (0.05 pmol) was used in the different incubations. Lanes a, b, c: the hexadecamer incubated without (a), with gene A protein (b) or with A* protein (c). Lanes d, e, f: the mixture of oligonucleotides obtained after partial digestion with snake venom phosphodiesterase and incubation without (d), with gene A protein (e) or with A* protein (f). Lanes g, h, i: the mixture of oligonucleotides obtained after partial digestion with spleen phosphodiesterase and labelling at the 5' end, followed by incubation without (g), with gene A protein (h) or with A* protein (i).

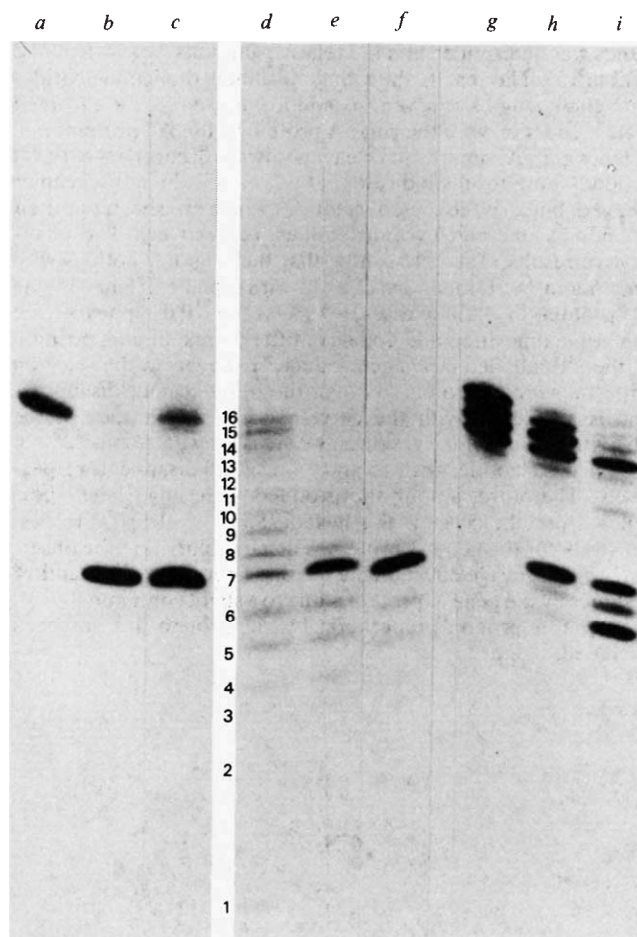
The nucleotide sequence of the synthetic DNA was confirmed using two different methods. The DNA was labelled at the 5' end with ³²P and partially digested with snake venom phosphodiesterase from the 3' end. The resulting oligonucleotides were separated by the standard two-dimensional system¹⁷. From the shifts in mobility of the successive radioactive products, a nucleotide sequence can be deduced (Fig. 1a, b) which is consistent with the one expected. The 5'-labelled synthetic DNA was also subjected to the chemical method for sequencing DNA^{18,19}. Again the sequence deduced from the successive bands in the autoradiograph (Fig. 1c) is consistent with that expected. However, from the intensities of the second slowest bands in the five lanes it can be inferred that a minor part of the hexadecamer has a deoxyguanosine instead of a deoxyadenosine as the 3'-terminal building block.

The ΦX gene A protein and A* protein nick the hexadecamer

The 5'-³²P-labelled hexadecamer was incubated with the purified ΦX gene A protein or the A* protein. Analysis of the reaction mixtures by polyacrylamide gel electrophoresis (Fig. 2, lanes a-c) shows that the mobility of a large portion of the labelled DNA is now present at a position corresponding to an oligonucleotide with a chain length of 7. This indicates that the gene A protein as well as the A* protein cleave the hexadecamer at the 3' side of the G residue, that is, at the same site at which gene A protein cleaves ΦX RFI DNA⁴ as well as ΦX single-stranded DNA¹². The A* protein also cleaves ΦX single-stranded DNA at this site although other sites are also cleaved¹². Some minor products with mobilities corresponding to chain lengths between 11 and 10 and between 10 and 9 nucleotides can also be observed (Fig. 2, Table 1a). We do not know how these minor products arise nor what they represent. To obtain extensive cleavage, the same high molar ratio (10-20:1) of enzyme to substrate is required as in the reaction with ΦX RFI DNA^{4-6,12}.

Which part of the hexadecamer is required for cleavage by gene A protein or A* protein?

First, it was determined which nucleotides from the 3' end of the hexadecamer are dispensable for cleavage by the gene A protein or the A* protein. Therefore, the synthetic DNA was labelled with ³²P at the 5' end and partially degraded by snake venom phosphodiesterase to form a mixture of oligonucleotides ranging from 1 to 16 nucleotides in length, all having the same 5' end.



This mixture was exposed to the gene A protein or the A* protein, respectively, and the products were analysed by polyacrylamide gel electrophoresis. The results (Fig. 2) show that a clear separation of the 16 oligonucleotides is obtained (lane d, control), and that both the gene A protein (lane e) and the A* protein (lane f) cleave the DNAs containing 16 to 10 nucleotides whereas the DNAs containing ≤9 nucleotides remain intact. The increase of DNA containing seven nucleotides (lanes e, f) indicates that cleavage occurs at the 3' side of the G residue in all oligonucleotides which are susceptible to cleavage by the gene A protein and the A* protein, respectively. These conclusions were confirmed by the determination of Cerenkov counts in the radioactive bands of the polyacrylamide gel (Fig. 2). As shown in Table 1a, the radioactivity of the oligonucleotides with lengths of 16 to 10 nucleotides decreases after incubation with gene A protein. The only significant increase in radioactivity is observed for the oligonucleotide containing seven nucleotides. The radioactivity of the other oligonucleotides does not differ significantly after incubation with or without gene A protein.

From the results in Fig. 2 and Table 1a, we conclude that the decamer CAACTTGATA is still cleaved, next to the G residue, by the gene A protein or the A* protein. Cleavage no longer takes place on further degradation from the 3' end, indicating that cleavage is specified by no more than the three nucleotides ATA behind the cleavage site.

The following experiment established which nucleotides from the 5' end of the hexadecamer are dispensable for cleavage by the gene A protein or A* protein. The synthetic DNA was degraded partially from the 5' end by incubation with spleen phosphodiesterase. In the resulting mixture the oligonucleotides have different 5' ends but the same 3' end. They were then ³²P-labelled at their 5' ends and incubated with gene A protein or A* protein. The radioactive products of these reactions were analysed by electrophoresis on a polyacrylamide gel and compared with the undigested mixture of oligonucleotides

Fig. 2, lanes *g-i*). The Cerenkov radiation from the radioactive bands was determined and the relative amounts were calculated (Table 1*b*). The results show that ^{32}P -labelled oligonucleotides with chain lengths of seven, six and five nucleotides are formed after incubation with the gene A protein or the A^* protein (Fig. 2, lanes *g-i*). Assuming that cleavage always occurs next to the G residue, these results indicate that not only is the hexadecamer cleaved, but also the oligonucleotides which are shorter at their 5' ends by one and two nucleotides, respectively. The quantitative results (Table 1*b*) show that the oligonucleotides with 16, 15 and 14 residues are cleaved with equal efficiency by the A^* protein. In all three cases, ~70% of the ^{32}P disappears from the original position and appears at the corresponding position of the ^{32}P -labelled cleavage product. However, in the reaction with the gene A protein, 69% of the hexadecamer disappears and is associated with the corresponding appearance of the oligonucleotide with 7 residues, whereas only 35% and 22% of the oligonucleotides with 15 and 14 residues disappear, respectively. Therefore, among the products which had been shortened from the 5' end, the hexadecamer is clearly the best substrate for the gene A protein. The possibility that the observed small cleavage effect on the oligonucleotides with 15 and 14 residues by the gene A protein is due to a slight contamination of the gene A protein preparation by A^* protein has not been excluded.

The results show, however, that at least the five nucleotides ACTTG before the cleavage site, are necessary for cleavage. Together with the observation that the three nucleotides ATA after the cleavage site are indispensable, this suggests that the minimal substrate for gene A protein and A^* protein is the octamer ACTTGATA.

This octamer was synthesized using the phosphotriester method^{15,16}. It was labelled at the 5' end and the nucleotide sequence determined using the two different methods as described for the hexadecamer (Fig. 3*a-c*). The sequence deduced from both analyses is consistent with that expected: ACTTGATA. Incubation of the octamer with the gene A protein did not result in an altered migration of the radioactive starting material (Fig. 4*A*, lanes *c, d*). Control experiments with the hexadecamer (Fig. 4*A*, lanes *a, b*) and a mixture of the hexadecamer and the octamer (Fig. 4*A*, lanes *e, f*) clearly show that the gene A protein was active and cleaves the hexadecamer. Analysis after incubation of the octamer with the A^* protein (Fig. 4*B*, lanes *c, d*) shows that the A^* protein cleaves the octamer. The resulting product is five nucleotides long, so A^* protein cleaves the octamer as expected, at the 3' side of the G residue. Control experiments with the hexadecamer (Fig. 4*B*, lanes *a, b*) and a mixture of the hexadecamer and the octamer (Fig. 4*B*, lanes *i, j*) show that the hexadecamer is a much better substrate for the A^* protein than the octamer. In the mixture of

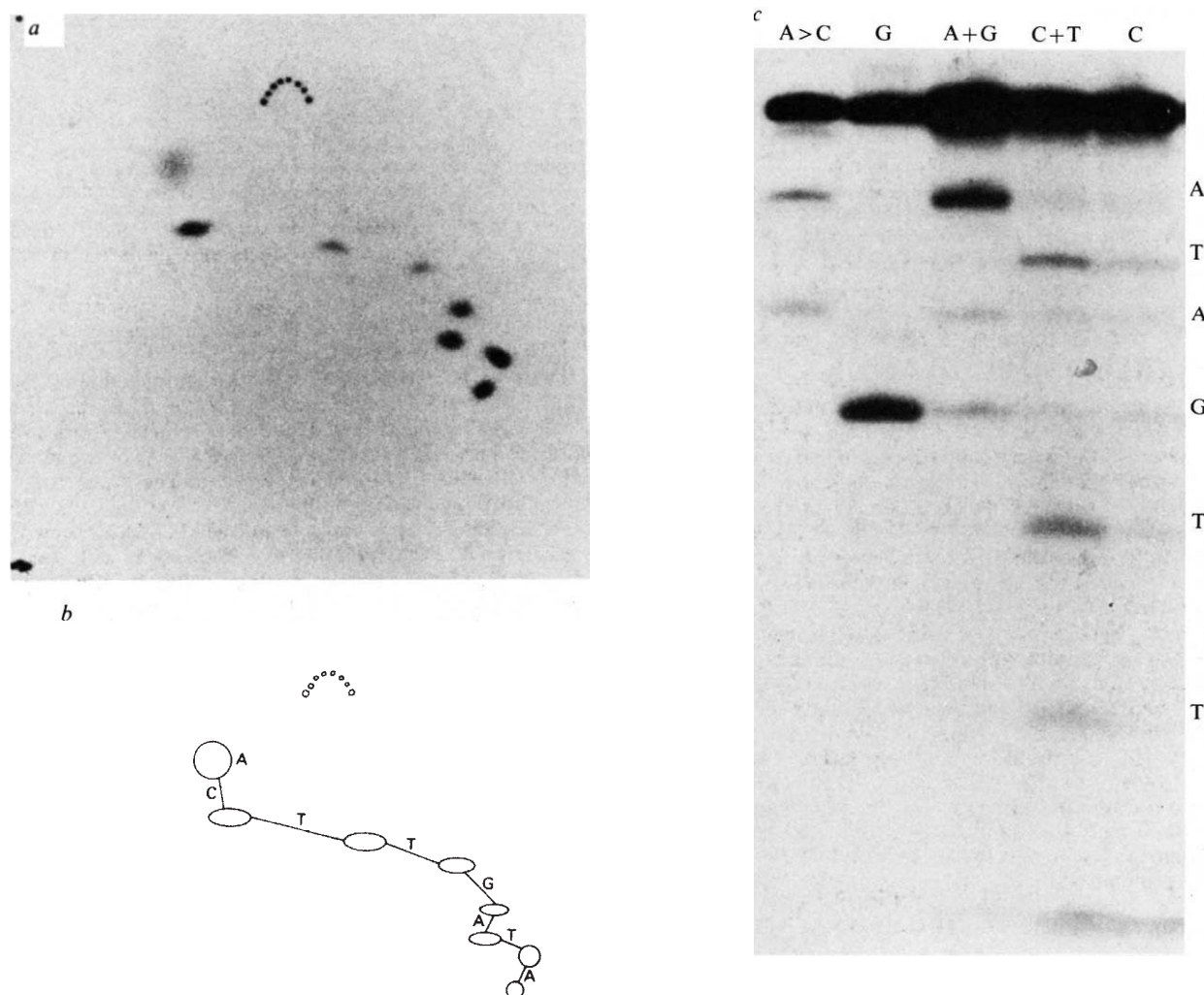


Fig. 3 Characterization of the synthetic octamer. The synthetic octamer was labelled at the 5' end and subjected to the different sequence analyses as described for the hexadecamer in Fig. 1 legend. *a*, The autoradiograph of the radioactive products obtained by partial digestion with snake venom phosphodiesterase and separated by electrophoresis and homochromatography. *b*, The interpretation of the autoradiograph. The capitals indicate which nucleotide causes the shift of an oligonucleotide to the next longer one. *c*, The autoradiograph of the sequencing gel, aligned with the sequence deduced from the successive bands.

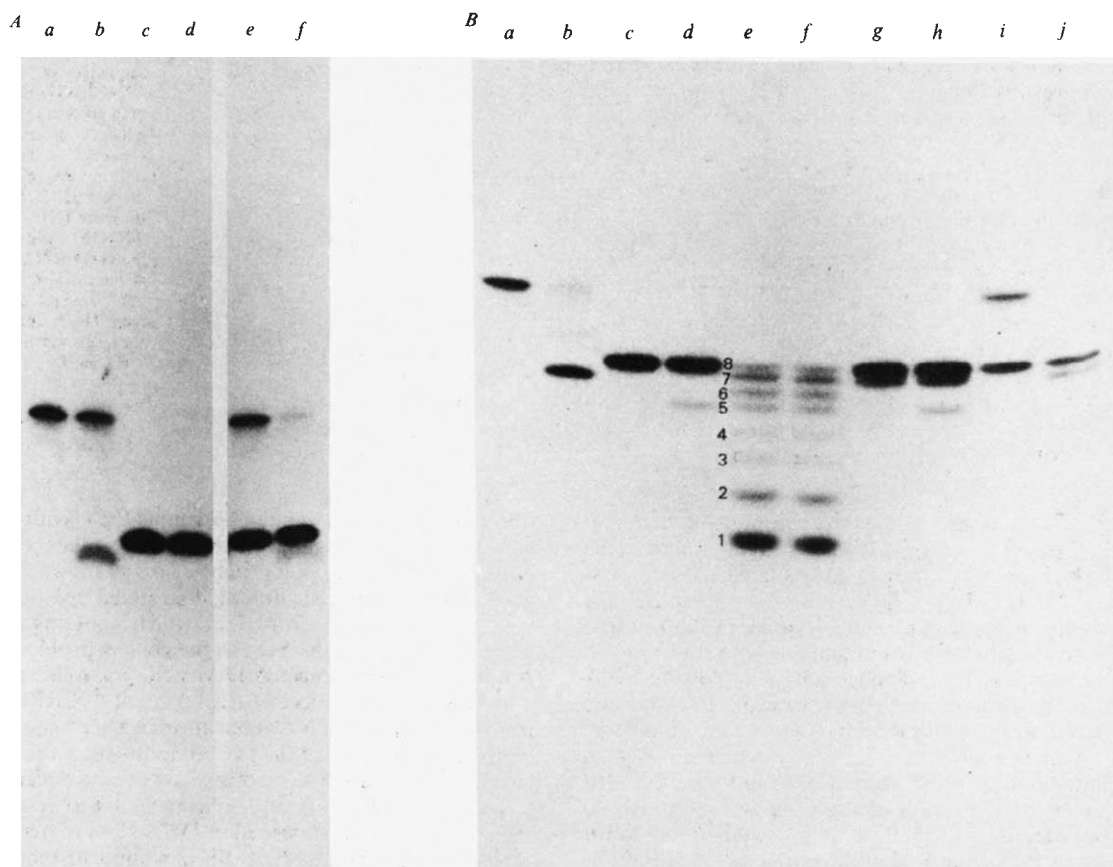


Fig. 4 The nicking activity of the gene A protein and A* protein on the octamer. 5'-Labelled octamer or hexadecamer (0.045 pmol) was incubated with gene A protein or A* protein as described in Fig. 2 legend. The mixtures of oligonucleotides, which had been shortened from the 3' end, or the 5' end, were prepared with snake venom phosphodiesterase or spleen phosphodiesterase as described in Figs 1 and 2 legends respectively. **A**, Lanes a, b: the hexadecamer after incubation without (a) and with (b) gene A protein. Lanes c, d: the octamer after incubation without (c) and with (d) gene A protein. Lanes e, f: the mixture of the hexadecamer and the octamer after incubation without (e) and with (f) gene A protein. **B**, Lanes a, b: the hexadecamer after incubation without (a) and with (b) A* protein. Lanes c, d: the octamer after incubation without (c) and with (d) A* protein. Lanes e, f: the mixture of oligonucleotides after partial digestion with snake venom phosphodiesterase after incubation without (e) and with (f) A* protein. Lanes g, h: the mixture of oligonucleotides obtained after partial digestion with spleen phosphodiesterase and labelling of the 5' end after incubation without (g) and with (h) A* protein. Lanes i, j: the mixture of the hexadecamer and the octamer after incubation without (i) and with (j) A* protein.

oligonucleotides obtained after partial digestion of the octamer from the 3' end with snake venom phosphodiesterase, the A* protein did not cleave a significant amount of any of the oligonucleotides shorter than eight nucleotides (Fig. 4B, lanes e, f). The two products obtained after partial digestion with spleen phosphodiesterase and labelling of the different 5' ends show that only the octamer is cleaved, resulting in the simultaneous appearance of an oligonucleotide with a chain length of five. The heptamer is not nicked by A* protein (Fig. 4B, lanes g, h).

Conclusions

The gene A protein cleaves the synthetic hexadecamer CAACTTGATATTAATA at the 3' side of the G residue, at which site the corresponding sequences in ΦX single-stranded DNA¹² and RFI DNA⁴ are also cleaved. Stepwise degradation from the 3' end shows that the decamer CAACTTGATA is cleaved by the gene A protein with about equal efficiency to the hexadecamer (Fig. 2, Table 1a). However, elimination of two residues from the 5' end greatly reduces, although it does not completely prohibit, cleavage by the gene A protein (Fig. 2, Table 1b). This suggests that the actual recognition sequence of the gene A protein is shorter than the decamer, possibly the octamer sequence ACTTGATA. This possibility is not inconsistent with the result that the gene A protein does not cleave the synthetic octamer. Similar conclusions were reached from studies on the nuclease activity of the restriction endonucleases *EcoRI*^{20,21}, *HpaII* and *MnoI*²², using short synthetic DNAs.

It is unlikely that sequences shorter than an octamer sequence can serve as the recognition site of the gene A protein. This conclusion is based on the fact that all possible sequences which have an uninterrupted stretch of seven nucleotides in common with the decamer CAACTTGATA occur in ΦX DNA and M13 DNA. These viral, single-stranded DNAs are not cleaved at these sites^{12,23,24} (CAACTTG, nucleotides 1,966–1,972 in ΦX DNA⁷; AACTTGA, nucleotides 2,492–2,498 and 5,695–5,701 in M13 DNA²⁵; ACTTGAT, nucleotides 2,493–2,499 and 5,696–5,702 in M13 DNA²⁵; CTTGATA, nucleotides 68–74 in ΦX DNA⁷).

In contrast to the gene A protein, the A* protein cleaves the octamer ACTTGATA. This indicates that the octamer sequence represents the maximum recognition sequence of the A* protein. The actual recognition sequence is probably less than eight, because the A* protein has been shown to cleave single-stranded ΦX DNA at about 30 different sites¹².

The possibility that gene A protein is considered to be a nuclease which acts specifically on single-stranded DNA has already been suggested by the finding that the gene A protein nicks supercoiled ΦX RFI DNA but not relaxed ΦX RFI DNA^{6,23,26}. Supercoiled DNA has been shown to contain regions of local unwinding, particularly at A + T-rich regions²⁷. Interestingly, in the RFI DNAs that are nicked by the gene A protein, the cleavage site is always located in an A + T-rich area (Fig. 5). It is thought that the gene A protein can nick these RFI DNAs, because the recognition sequence is present in a (partially) single-stranded form. However, supercoiled ΦX RFI DNA is not nicked by the A* protein^{12,14,23}, indicating that its

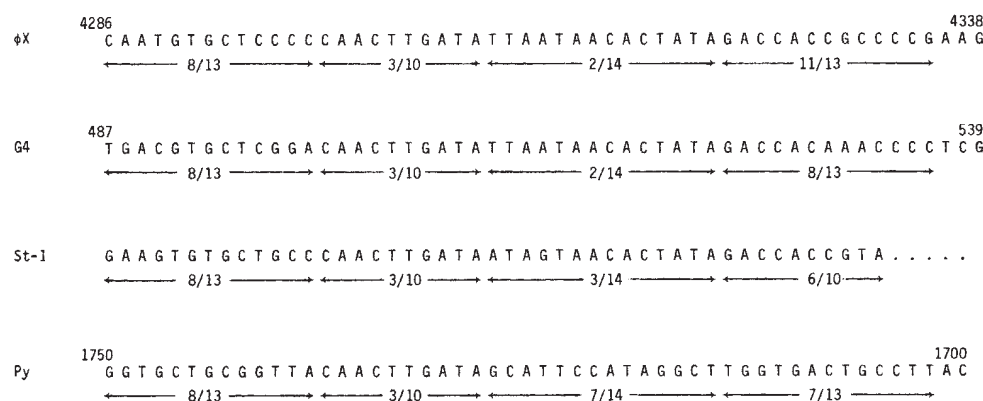


Fig. 5 The nucleotide sequence of the DNA of ΦX174, G4, St-1 and polyoma virus in the region of the gene A protein recognition sequence. The fractions indicate the relative amounts of G and C residues in the different DNA stretches. The ΦX DNA sequence is from Sanger *et al.*⁷, the G4 DNA sequence from Godson *et al.*³⁶, the St-1 DNA sequence from Heidekamp *et al.*¹⁰ and the polyoma DNA sequence is from Deininger *et al.*³⁴.

recognition sequence is not sufficiently exposed in a single-stranded form. Thus the longer recognition sequence of the gene A protein may be expected to show even less single strandedness in supercoiled ΦX RFI DNA. Nevertheless, supercoiled ΦX RFI DNA is efficiently nicked by the gene A protein. These considerations lead to the conclusion that the gene A protein, in contrast to the A* protein, can cause unwinding of the DNA double helix at the recognition sequence, which can then be nicked. The unwinding could be driven by the release of (part of) the free energy of the superhelix formation which could take place during the binding of the gene A protein to the ΦX RFI DNA. This unwinding property of the gene A protein is also suggested from the observation that the protein is required for the movement of the replication fork during ΦX DNA replication *in vitro*^{5,6}. It seems likely that the DNA unwinding properties of the gene A protein are located in the N-terminal part of the protein chain which is not present in the A* protein.

The decamer sequence CAAGTGTGCTGCTGCGGTTTCACTTGTATAGCATTCCATAGGCTTGGTGAAGTGCCTTAC is unique in ΦX DNA⁷. It does not occur in M13 DNA²⁵, fd DNA²⁸, SV40 DNA^{29,30}, BKV DNA³¹ or pBR322 DNA³². However, in polyomavirus DNA, the sequence CAAGTGTGCTGCTGCGGTTTCACTTGTATAGCATTCCATAGGCTTGGTGAAGTGCCTTAC occurs (nucleotides 1,719–1,710 (ref. 33) or 1,737–1,728 (ref. 34)) in the strand with the same polarity as the late mRNAs³³. The supercoiled polyoma virus component I DNA is not nicked by the gene A protein whereas St-1 RFI DNA in the same reaction mixture is nicked efficiently (F. Heidekamp, personal communication). Comparison of the DNA sequence surrounding CAAGTGTGCTGCTGCGGTTTCACTTGTATAGCATTCCATAGGCTTGGTGAAGTGCCTTAC in polyoma DNA with that of the DNAs of the isometric phages ΦX, G4 and St-1 (Fig. 5) indicates that a characteristic A + T-rich region which is present in the isometric phage DNAs is absent in polyoma DNA. This may explain the

observation that supercoiled polyoma DNA is not nicked by the gene A protein. In single-stranded DNA the gene A protein does not require the A + T-rich region as shown by the present work and the fact that the single-stranded polyomavirus DNA restriction fragment *Hae*III 8, which contains the sequence CAAGTGTGCTGCTGCGGTTTCACTTGTATAGCATTCCATAGGCTTGGTGAAGTGCCTTAC, is cleaved by the gene A protein next to the G residue in this sequence (A.D.M.v.M., unpublished results).

In summary, the successful reaction of ΦX RFI DNA and its initiator protein of DNA replication has been shown to depend on a subtle interplay of the two components of the reaction. The substrate requirements involve (part of) the decamer sequence CAAGTGTGCTGCTGCGGTTTCACTTGTATAGCATTCCATAGGCTTGGTGAAGTGCCTTAC and an adjacent A + T-rich region, which must be present in supercoiled DNA. In supercoiled ΦX RFI DNA this region is sufficiently unwound to bind the gene A protein. This binding causes local unwinding of the DNA double helix which exposes the recognition sequence in a single-stranded form. Subsequently, the gene A protein cleaves one strand, the strand containing the sequence CAAGTGTGCTGCTGCGGTTTCACTTGTATAGCATTCCATAGGCTTGGTGAAGTGCCTTAC. Cleavage occurs at the 3' side of the G residue, and creates a primer for DNA polymerase III holoenzyme to start the rolling circle DNA replication⁵.

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- Sinsheimer, R. L., Starman, B., Nagler, C. & Guthrie, S. *J. molec. Biol.* **4**, 142–160 (1962).
- Francke, B. & Ray, D. S. *J. molec. Biol.* **61**, 565–586 (1971).
- Baas, P. D., Jansz, H. S. & Sinsheimer, R. L. *J. molec. Biol.* **102**, 633–656 (1976).
- Langeveld, S. A. *et al. Nature* **271**, 417–420 (1978).
- Eisenberg, S., Griffith, J. & Kornberg, A. *Proc. natn. Acad. Sci. U.S.A.* **74**, 3198–3203 (1977).
- Ikeda, J. E., Yudelevich, A. & Hurwitz, J. *Proc. natn. Acad. Sci. U.S.A.* **73**, 2669–2673 (1976).
- Sanger, F. *et al. J. molec. Biol.* **125**, 225–246 (1978).
- Eisenberg, S. & Kornberg, A. *J. biol. Chem.* **254**, 5328–5332 (1979).
- van Mansfeld, A. D. M. *et al. Cold Spring Harb. Symp. quant. Biol.* **43**, 331–334 (1979).
- Heidekamp, F., Langeveld, S. A., Baas, P. D. & Jansz, H. S. *Nucleic Acids Res.* **9**, 2009–2021 (1980).
- Duguet, M., Yarranton, G. & Gefter, M. *Cold Spring Harb. Symp. quant. Biol.* **43**, 335–343 (1979).
- Langeveld, S. A., van Mansfeld, A. D. M., de Winter, J. M. & Weisbeek, P. J. *Nucleic Acids Res.* **8**, 2177–2188 (1979).
- Linney, E. A. & Hayashi, M. N. *Nature new Biol.* **245**, 8–9 (1973).
- Langeveld, S. A., van Arkel, G. A. & Weisbeek, P. J. *FEBS Lett.* **114**, 269–272 (1980).
- Arentzen, R., van Boeckel, C. A. A., van der Marck, G. & van Boom, J. H. *Synthesis* **137**–139 (1979).
- de Rooij, J. F. M., Wille-Hazeleger, G., van Deursen, P. H., Serdijn, J. & van Boom, J. H. *Recl. Trav. Chim. Pays-Pas* **98**, 537–548 (1979).
- Brownlee, G. G. & Sanger, F. *Eur. J. Biochem.* **21**, 395–399 (1969).

- Maxam, A. M. & Gilbert, W. *Proc. natn. Acad. Sci. U.S.A.* **74**, 560–564 (1977).
- Maxam, A. M. & Gilbert, W. *Meth. Enzym.* **65**, 499–559 (1980).
- Greene, P. J. *et al. J. molec. Biol.* **99**, 237–261 (1975).
- Goppelt, M., Pingoud, A., Maass, G., Köster, H. & Frank, R. *Eur. J. Biochem.* **104**, 101–107 (1980).
- Baumstark, B. G., Roberts, R. J. & Raj Bhandary, U. L. *J. biol. Chem.* **254**, 8943–8950 (1979).
- Ikeda, J. E., Yudelevich, A., Shimamoto, N. & Hurwitz, J. *J. biol. Chem.* **254**, 9416–9428 (1979).
- Henry, T. J. & Knippers, R. *Proc. natn. Acad. Sci. U.S.A.* **71**, 1549–1553 (1974).
- van Wezenbeek, P. M. G. F., Hulsebos, T. J. M. & Schoenmakers, J. G. G. *Gene* **11**, 129–148 (1980).
- Marians, K., Ikeda, J. E., Schlagman, S. & Hurwitz, J. *Proc. natn. Acad. Sci. U.S.A.* **74**, 1965–1968 (1977).
- Jacob, R. J., Lebowitz, J. & Kleinschmidt, A. K. *J. Virol.* **13**, 1176–1185 (1974).
- Beck, E. *et al. Nucleic Acids Res.* **5**, 4495–4503 (1978).
- Reddy, V. B. *et al. Science* **200**, 494–502 (1978).
- Fiers, W. *et al. Nature* **273**, 113–120 (1978).
- Yang, R. C. A. & Wu, R. *Science* **206**, 456–462 (1979).
- Sutcliffe, J. G. *Cold Spring Harb. Symp. quant. Biol.* **43**, 77–90 (1979).
- Soeda, E., Arrand, J. L., Smolar, N., Walsh, J. E. & Griffin, B. E. *Nature* **283**, 445–453 (1980).
- Deininger, P. L., Esty, A., LaPorte, P., Hsu, H. & Friedmann, T. *Nucleic Acids Res.* **8**, 855–860 (1980).
- van Mansfeld, A. D. M., Vereijken, J. M. & Jansz, H. S. *Nucleic Acids Res.* **3**, 2827–2844 (1976).
- Godson, G. N., Barrell, B. G., Staden, R. & Fiddes, J. C. *Nature* **276**, 236–247 (1978).

LETTERS

Transition to oscillatory motion in the Taylor experiment

T. Mullin & T. Brooke Benjamin

Mathematical Institute, 24–29 St Giles, Oxford OX1 3LB, UK

Debates about hydrodynamic stability and the origins of turbulence are often based on the phenomena observable in Taylor's experiment¹ on the flow between concentric circular cylinders, the inner of which rotates while the outer is stationary. To reappraise a central aspect of this subject, we report here the effects of annulus length on the limit of stability for various steady cellular flows. For comparatively small values of the aspect ratio Γ , unforeseen behaviour has been observed indicating enormous sensitivity to end effects. In particular, plots of critical Reynolds number R_w against Γ are sharply peaked, and the form of oscillations arising at the stability limit changes in successive ranges of Γ . The new phenomena are beyond the reach of any present theory, and they highlight the need for caution when transition observations are interpreted.

The variety of phenomena observable in the Taylor experiment can be classified in terms of a Reynolds number R whose value fixes dynamical similarity for given proportions of the annular space filled by the fluid: say, $R = \Omega r_1 d / \nu$, where Ω is the angular velocity of the inner cylinder, r_1 its radius, $d = r_2 - r_1$ the gap width and ν the kinematic viscosity of the fluid. At small R the flow has no detectable longitudinal or radial component. However, when R is raised into a narrow, quasi-critical range, typically < 100 , the flow develops a cellular structure, remaining steady and axisymmetric but being such that in adjacent cells the fluid particles move in counter-rotating spiral paths. When R is raised above a higher threshold value, the flow becomes unsteady with its cellular structure perturbed by circumferential travelling waves. As R increases further, the temporal variations become progressively more complex, exhibiting several incommensurate frequencies, and eventually a turbulent but perhaps still regularly pulsating state of motion is observed. Recent experimental advances^{2,3} have renewed interest in these later stages of the transition to turbulence. In particular, the Taylor experiment should be a test of new theoretical concepts⁴ concerning the incipience of turbulence.

Most theoretical studies of the Taylor experiment have been simplified by assuming the annulus to be infinitely long. These have given good estimates for the value of R at which cells first appear but little insight into the process whereby the number of cells is related to the annulus length. Earlier experiments⁵ have, however, shown that the steady cellular flows realizable in the second stage of the Taylor experiment are not unique, and that the length-dependent selection process is complex and quite beyond simulation by the idealized model. Moreover, the multiplicity of steady flows possible at sufficiently high R values includes various 'anomalous' modes which cannot, for any annulus length, be produced by gradually increasing R from small values⁶. Although these phenomena have all been qualitatively explained by abstract methods of analysis^{7,8}, they have not yet been covered by numerical or other constructive calculations. Non-uniqueness complicates the third, travelling-wave stage of the Taylor experiment⁹, and clearly the complicated solution set in this and the second stage will have to be elucidated further before the final transition to turbulence can be properly understood.

We have modified previous apparatus⁵ to give finer control of temperature in the fluid and of rotor speed. The end conditions are virtually symmetric, the bottom of the fluid-filled annular space being a fixed wall and the top being the surface of a PTFE collar whose distance l from the bottom is continuously adjust-

able from 0 to 126 mm. (Previous, otherwise comparable experiments have used a free upper boundary^{10,11}.) The radii of the cylindrical boundaries are $r_1 = 36.9$ mm and $r_2 = 60.0$ mm, so that the aspect ratio $\Gamma = l/d$ is variable from 0 to 5.45.

The working fluid for these experiments was an aqueous solution of glycerol, 50:50 by volume, to which a small quantity of a pearly substance was added for flow visualization. Illuminated laterally, the Taylor cells were seen through the transparent outer cylinder as spiralling myriads of light specks, separated by thin dark bands. The extremely sensitive method of visualization revealed both the finest details of a steady flow and the onset of travelling waves when R was gradually raised to its critical value. A periodic rise and fall of the cell boundaries could then be seen. When established, however, the time-dependent motions were too complex for their spatial forms to be readily appreciated by the unaided observer. For later measurements of wave speeds, a greater amount of the visualizing additive was used, although not enough to affect the mechanical properties of the liquid but still able to produce vivid images of the kind that has often been recorded^{2,9}. As a guide to the identification of angular wavenumbers among the different oscillations observed, photographs and a cine-film were used.

The production of various steady cellular flows in this apparatus, including the anomalous modes, has been described previously⁵. The stability of four such flows is now considered: (1) the two-cell mode in which the fluid spirals in the normal way, radially inwards near the end walls; (2) the normal four-cell mode; (3) the three-cell mode which is alternatively realizable with an abnormally spiralling cell at one end or the other; and (4) the abnormal four-cell mode in which each cell spirals oppositely to its counterpart in (2). The main observations involved establishing a particular mode in a steady state for a measured annulus length l , and then raising the rotor speed in very small steps until travelling waves appeared. In this way the critical value R_w of R marking the limit of stability was found as a function of Γ . After each increment of speed, the system was allowed to settle into its respective steady state, and for consistent results in approaching critical speeds the settling times needed to be prolonged far beyond those suggested by Snyder's¹⁰ empirical estimate.

Having established travelling waves at constant amplitude, the rotor speed was then very gradually reduced, with the same precautions about adequate settling times, until the wavy motion ceased. A definite hysteresis, spanned by the interval from R_w down to a second critical value $R'_w < R_w$, was thus observable in most cases. This was manifested by the sudden cessation of previously vigorous oscillations, the hysteresis limit $R = R'_w$ could be confidently estimated by our visual method. The frequency of the waves, $f = \omega/2\pi$ Hz, was measured by timing a predetermined large number of periods with a stopwatch.

Figure 1 shows the results for R_w and R'_w plotted against Γ , where the most outstanding feature is the set of high peaks in the stability curves for the four different modes of steady axisymmetric flow. For each mode there is an optimal length of the annulus for which the stable range of rotor speeds is most prolonged, and the maximal values of R_w are surprisingly great. For the two normal modes, these values are in fact ~ 18 times the critical value of R for the first appearance of cells according to the idealized theory, and ~ 15 times the estimate of R_w on a similar basis ignoring end effects¹². Cole¹¹ has also observed rapid increases in R_w , to more than double the estimated value, on reductions of aspect ratio Γ below ~ 10 . (His results, however, do not make it clear whether the same cellular mode had been maintained as Γ was varied.) On the other hand, the well defined optimal length for each mode is quite unexpected, and its distinctness suggests that it should not be exclusive to the modes with comparatively few cells.

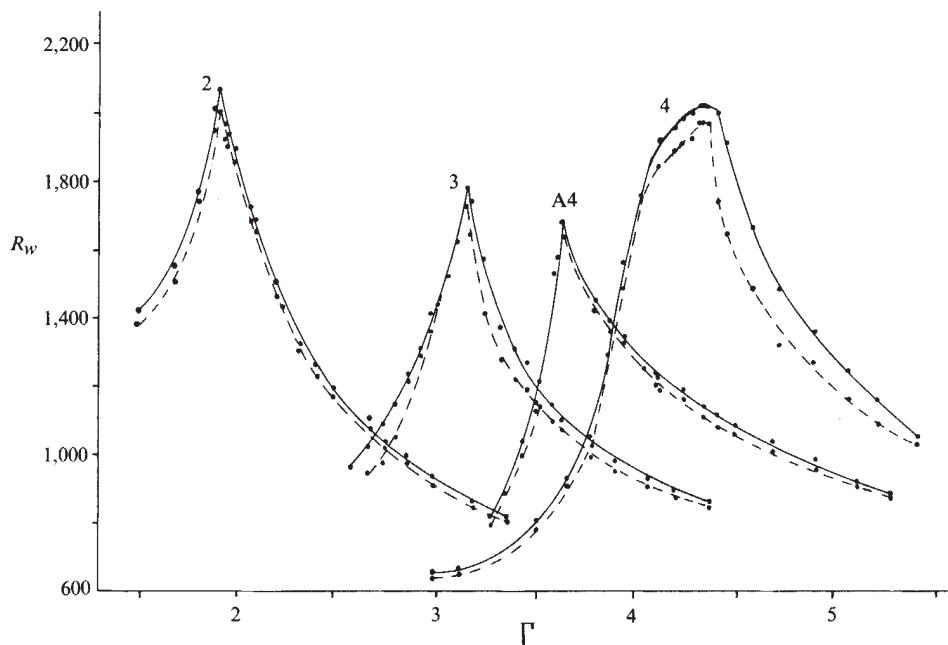


Fig. 1 Stability limit R_w and hysteresis limit R'_w as a function of aspect ratio Γ , for four cellular modes.

The curious ridge broadening the peak in the plot of R_w against Γ for the normal four-cell mode is associated with occurrence of travelling waves that have azimuthal wave number $n = 1$ (that is, period 2π in the cylindrical polar angle). This 'weak wavy mode', so called by Markho *et al.*¹³, was observed only in this region of Fig. 1. For settings $\Gamma < 4.10$, to the left of the ridge, the instability of the normal four-cell mode was manifested as waves with $n = 2$, giving rise to periodic oscillations of the upper and lower intercellular boundaries but leaving the central boundary steady. With Γ raised into the range 4.10–4.34, however, the unusual wave-form having $n = 1$ arose; then oscillations appeared on the central interface but none on the other two. After a further increase of Γ into the range 4.34–4.72 which includes the optimal value, the onset of unsteadiness took the more complicated form of a quasi-periodic motion exciting all four cells, which was comprised from waves with $n = 1$ and those with $n = 5$ whose frequency was much higher. This form of motion was visibly quite distinct from the singly periodic motions observed elsewhere. For $\Gamma > 4.72$, a singly periodic motion was again encountered, but in this case with $n = 5$.

The two-cell mode was observed to follow a simpler pattern of behaviour at its stability locus as shown in Fig. 1. It had access there only to periodic waves with either $n = 3$ or $n = 5$, depending on Γ .

The peaks of R_w for the two anomalous modes are ~12% and 22% lower than those for the normal modes, and this deficiency seems consistent with the comparatively high lower limits also set on their ranges of stability⁶. Over a range of Γ upwards from just below the respective optimal value, each of these modes developed singly periodic travelling waves with $n = 5$ when R had been raised gradually to R_w . In common with the normal

modes, however, they could be switched to wave regimes with other values of n by means of sudden changes in the rotor speed. Over this range each anomalous mode had the striking property that, after the onset of travelling waves, the normally spiralling cells executed distinct oscillations whereas none was observable in the abnormal cells (at one end for the three-cell mode, and at both ends for the anomalous four-cell mode). In fact, for the four-cell mode, increases in speed beyond the threshold value produced a multiply-periodic wave system disturbing the central interface but still causing no observable oscillations in the end cells.

Over a range to the left of the respective peak in Fig. 1, on the other hand, each anomalous mode exhibited another category of time-dependent motion when R had been raised to R_w . The motions were seen to be irregular, featuring slow pulsations of large amplitude but without any well defined low frequency. Because there were some moderately energetic components at higher frequencies, the observed motions might perhaps qualify as a primitive form of turbulence. However, their lack of resemblance to any familiar example of turbulence, including flows in our apparatus at much higher R , makes us wary of such a description.

For conditions at the left-hand end of Fig. 1, the irregular motions grew large enough to cause coalescence of the central and abnormal end cells, whereupon the three-cell mode collapsed into the two-cell mode which duly became steady. A similar situation, where the onset of waves resulted in the eventual collapse of the flow structure, was found for the abnormal four-cell mode in a range of Γ , 3.52–4.07, to the left of the peak in the experimental graph A4. This collapse was into the three-cell mode, which became steady or mildly wavy depending on Γ . Spontaneous mode-jumping in this manner has been described by Coles⁹, referring to arrays of many Taylor cells, but none of his observations exemplified the present case of collapse from an oscillating mode into a steady one.

Most of the observed transitions to time-dependent states of flow evinced appreciable hysteresis, as shown by the experimental plots of R'_w against Γ (dashed curves in Fig. 1). An interesting feature of the graphs is that the amount of hysteresis, $R_w - R'_w$, varies quite abruptly with Γ in certain places, which correspond to the changes that were observed among the types of wavy motion arising at $R = R_w$. This feature is particularly prominent near the peak of the graphs for the normal four-cell mode. It had seemed plausible that the irregular, multi-component oscillations of the anomalous modes at annulus lengths less than the optimal would have a large hysteresis, and

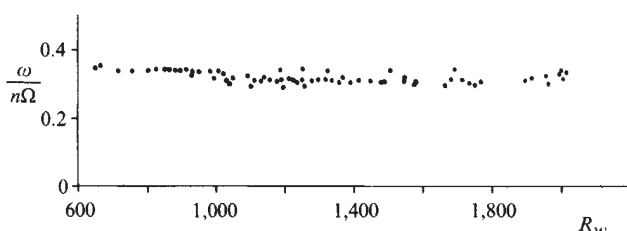


Fig. 2 Dimensionless angular speed of travelling waves that appear at stability limit R_w .

that as R was reduced they might first relax into periodic oscillations before the unsteady motion ceased at $R = R'_w$; but no such behaviour was detected experimentally.

The transition has great sensitivity to end effects; but in contrast another, more easily recorded property was found to be surprisingly insensitive to them. Expressed as a multiple of Ω , the angular speed ω/n of the observed travelling waves turned out to be nearly independent of R , as shown in Fig. 2 which includes values of $\omega/n\Omega$ from all our measurements of frequency. The mean value is about 0.32, which is unexpectedly close to corresponding values found previously by others from observations in apparatus with Γ an order of magnitude larger, for example the value 0.34 found by Coles⁹. Considered alone, this property might thus be deceptive as regards the overall importance of end effects.

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1. Taylor, G. I. *Phil. Trans. R. Soc. A* **223**, 289–343 (1923).
2. Fenstermacher, P. R., Swinney, H. L. & Gollub, J. P. *J. Fluid Mech.* **94**, 103 (1979).
3. Walden, R. W. & Donnelly, R. J. *Phys. Rev. Lett.* **42**, 301–304 (1979).
4. Ruelle, D. & Takens, F. *Commun. Math. Phys.* **20**, 167–192 (1971).
5. Benjamin, T. B. *Proc. R. Soc. A* **359**, 27–43 (1978).
6. Benjamin, T. B. & Mullin, T. *Phil. Trans. R. Soc. A* (submitted).
7. Benjamin, T. B. *Proc. R. Soc. A* **359**, 1–26 (1978).
8. Schaeffer, D. G. *Math. Proc. Camb. Phil. Soc.* **87**, 307–337 (1980).
9. Coles, D. *J. Fluid. Mech.* **21**, 385–425 (1965).
10. Snyder, H. A. *J. Fluid. Mech.* **35**, 273–298 (1969).
11. Cole, J. A. *J. Fluid. Mech.* **75**, 1–15 (1976).
12. Eagles, P. M. *J. Fluid. Mech.* **49**, 529–550 (1971).
13. Markho, P. H., Jones, C. D. & Mobbs, F. R. *J. Mech. Engng Sci.* **19**, 76–80 (1977).

Complete vitrification in pure liquid water and dilute aqueous solutions

Peter Bruggeller & Erwin Mayer

Institut für Anorganische und Analytische Chemie, Universität Innsbruck, A 6020 Innsbruck, Austria

Pure water can only be vitrified by the very slow condensation of vapour on a metal surface maintained at very low temperatures^{1,2}. Attempts to form vitreous ice by rapid cooling of liquid water invariably lead to formation of ice I_h (ref. 3). (Pryde and Jones⁴ did report a heat capacity change of rapidly cooled water at 126 K which they attributed to a glass transition, but could not reproduce this result in subsequent experiments.) Dilute aqueous solutions in contrast to concentrated aqueous solutions⁵ behave similarly to water and separate during freezing, even with the highest cooling rates available, into pure ice and concentrated solute⁶. We report here that macroscopic parts of samples of pure liquid water and of dilute aqueous solutions can be vitrified completely by jet-freezing of micrometre-sized aqueous droplets distributed in *n*-heptane as an emulsion—the resulting supercooling effect of ~40 K being essential for vitrification⁷.

The formation of vitrified ice from pure liquid water is demonstrated by the differential thermal analysis (DTA) warm-up curve of a jet-frozen emulsion of pure water (Fig. 1b). The exothermic peak at 150 K occurs at the temperature reported for the devitrification of amorphous ice prepared from water vapour⁸. The endothermic peak at 183 K is due to an *n*-heptane impurity. Jet-frozen 0.10 M CuCl_2 solutions (chosen for ESR requirements) gave similar warm-up curves with the exothermic peak also occurring at 150 K. We have not observed clearly a glass transition. Only a faint endothermic inclination at ~138 K in warm-up curves of jet-frozen water and of 0.10 M CuCl_2 solutions might come from the glass transition of the vitreous phase which compares well with the glass transition temperature of vapour deposited amorphous ice⁸. The glass transition will be unmistakable only in vitreous samples with little or no crystalline impurity, and investigations of vitreous ice formed from water vapour seem to confirm this⁸.

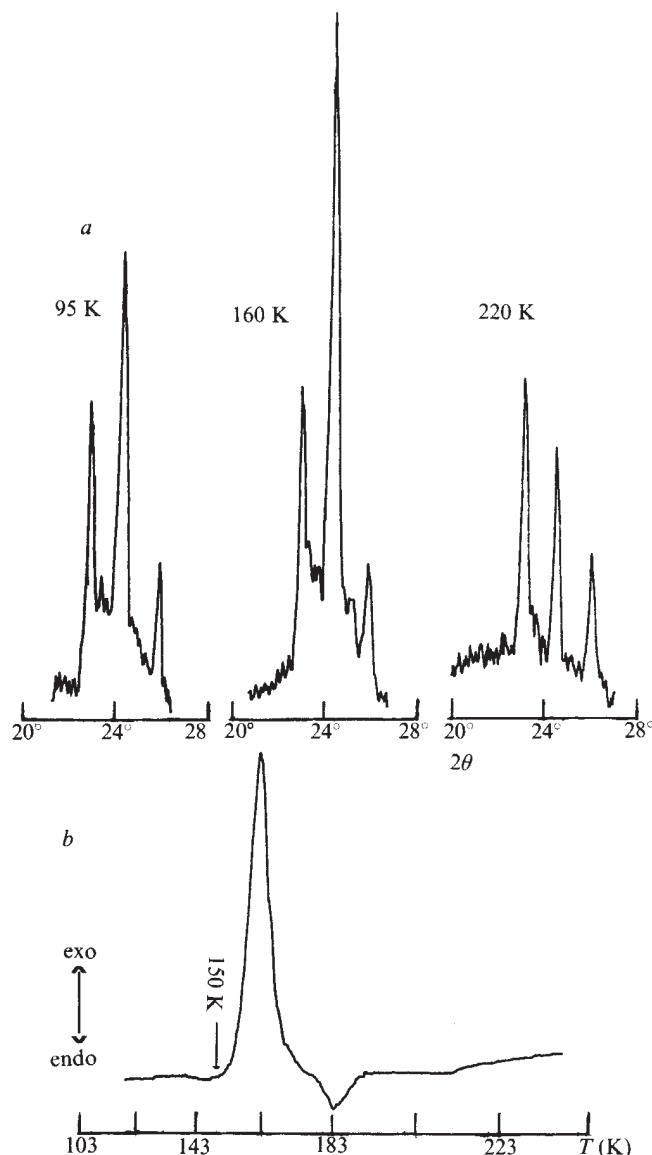


Fig. 1 *a*, X-ray diffraction pattern ($\text{Cu K}\alpha$) of a jet-frozen emulsified 0.10 M CuCl_2 solution, heated consecutively from 95 K to 160 and to 220 K. *b*, DTA warm-up curve of jet-frozen emulsified pure liquid water. Heating rate 6°min^{-1} . The low-temperature DTA apparatus was similar to that used by Rasmussen⁹. The NiCr-Konstantan combination was used as thermocouple and the bottom parts of thin-walled 10-mm diameter NMR tubes were used for sample and reference, and for temperature measurement. About 100 mg of sample were diluted with 300 mg CaCO_3 and transferred quickly into the precooled DTA apparatus. CaCO_3 was used as inert reference and in the tube for temperature measurement. Samples were prepared as follows: the emulsion was prepared by dissolving 0.030 g Span 65 (sorbitan tristearate) in 9 ml *n*-heptane, adding 1 ml pure liquid water (*b*) respectively 1 ml 0.10 M aqueous CuCl_2 solution (*a*) and sonifying the mixture. Droplet size depends strongly on sonifier energy and was adjusted to 1–5 μm (larger droplets supercool insignificantly⁷ and the ESR spectra indicated strong aggregation). 5 ml emulsion were jetted through a thick-walled glass tube with a 50 μm aperture, using 100 atm working pressure, into 120 ml of vigorously stirred liquid ethane cooled to ~90 K. We preferred ethane to propane as cryomedium because ethane can be pumped off quantitatively at 120 K. For DTA and X-ray experiments *n*-heptane was removed nearly completely by washing the frozen droplets several times with liquid ethane at 110 K. After pumping off the remaining ethane at 120 K the frozen droplets were transferred quickly with a liquid-nitrogen cooled spoon into the precooled apparatus. During the whole procedure the droplets were warmed at most to 130 K. At liquid-nitrogen temperatures special care has to be taken to prevent accumulation of liquid oxygen in the cryomedium.

The amount of the vitrified material was determined by calibration with a 50% w/v vitrified glycerol solution. Devitrification of a 50% w/v glycerol solution produces according to Rasmussen and Luyet^{9,10} a 73% w/v glycerol solution. We obtain an estimate by comparing the peak areas. To reduce errors both standard and sample were diluted equally with CaCO_3 and an amount of vitrified glycerol was chosen with a similar signal intensity to our vitrified samples. We found that jet-frozen water/heptane emulsions contain 13% vitrified ice. For jet-frozen emulsified 0.10 M CuCl_2 solutions a slightly higher value of 16% devitrified ice was obtained after several determinations. This only slightly higher value indicates that the freezing behaviour of a 0.10 M solution is comparable with the freezing behaviour of pure water, the small amount of additional devitrification coming from more concentrated vitrified parts of the solution.

We also investigated the vitrification of aqueous emulsions by X-ray diffraction and ESR spectroscopy. The X-ray diffraction pattern of ice allows us to differentiate between the phases I_h and I_c and mixtures of these, by the relative intensities of the three strong reflexes of ice I_h (for Cu $K\alpha$: $2\theta = 22.8^\circ$, 24.3° and 25.9°) and the strong reflex of ice I_c ($2\theta = 24.3^\circ$)^{11,12}. The presence of vitreous components in a partially crystalline sample can be followed by the formation of ice I_c at the devitrification temperature and the relative increase of the reflex of ice I_c at $2\theta = 24.3^\circ$. Figure 1a shows the characteristic part of the X-ray diffraction pattern of a jet-frozen 0.10 M CuCl_2 emulsion. The diffraction pattern at 95 K indicates a mixture of ice I_h and I_c . At 160 K a drastic increase of the reflex at $2\theta = 24.3^\circ$ indicates the formation of ice I_c by devitrification of an amorphous phase. At still higher temperatures, ice I_c transforms to ice I_h and at 220 K only the reflexes of I_h are visible. Our experimental conditions inevitably lead to contamination of the original jet-frozen sample by ice I_h due to condensation of water vapour during the preparation and transfer of the sample to the X-ray camera, therefore, the resulting X-ray diffraction pattern at 95 K will contain an unknown amount of ice I_h .

The freezing behaviour of the solute in a dilute solution was studied by ESR spectroscopy. Whereas most other spectroscopic techniques are only sensitive to short-range order (that is,

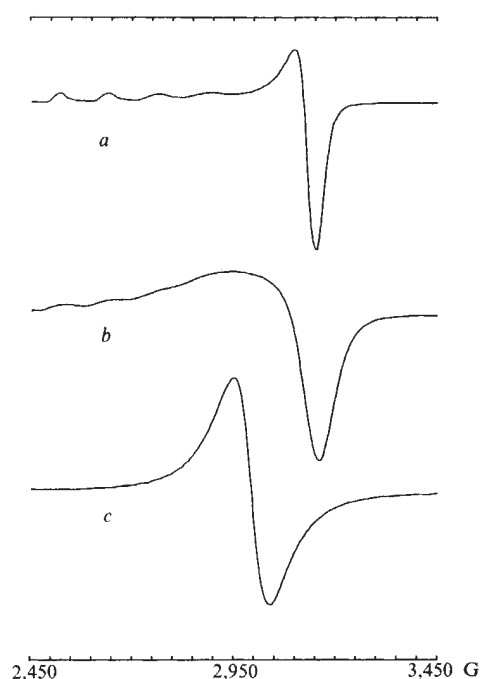


Fig. 2 Low temperature (113 K) ESR spectra of: *a*, 0.10 M CuCl_2 solution, containing 50% (v/v) glycerol for vitrification; *b*, 1.5 M CuCl_2 solution vitrified as above; *c*, 0.10 M CuCl_2 slowly frozen by immersion in liquid nitrogen.

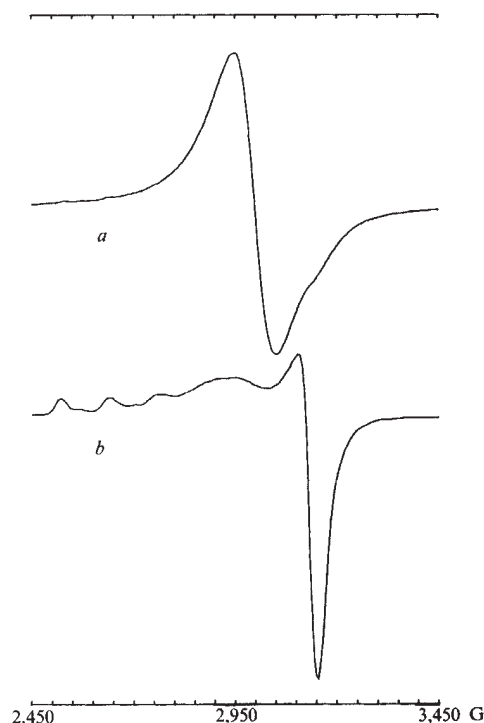


Fig. 3 Low temperature (113 K) ESR spectra of: *a*, 0.10 M CuCl_2 solution spray-frozen into liquid propane cooled to ~ 80 K. The reported technique¹⁵ was slightly modified: the solution was sprayed into 50 ml of stirred propane and the suspension filtered at 90 K. *b*, A jet-frozen 0.10 M CuCl_2 emulsion using optimized conditions as described in Fig. 1.

the first hydration sphere), ESR is sensitive to comparatively long range order effects due to the $1/r^3$ dependence of dipolar interaction¹³. With our system we can differentiate clearly between a 0.10 and a 1.5 M vitrified solution (Fig. 2*a,b*). We used ESR spectroscopy to maximize the various parameters such as cooling conditions and droplet size and found the spectra invaluable for assessing qualitatively the relative amounts of phase separated crystalline and of vitrified material. We used a 0.10 M aqueous CuCl_2 solution as our ESR test system because: (1) the spectra representing the two extremes—perfect vitrification and complete phase separation—are shown in Fig. 2*a* and *c* and are separated sufficiently for evaluation. (2) At a concentration of 0.10 M the ESR spectrum was the most sensitive to the segregation of ice during the freezing process. At higher concentrations the ESR signal begins to broaden due to dipole-dipole interactions as shown in Fig. 2*b*. At lower concentrations the spectra of glycerol vitrified solutions are nearly identical to the ESR spectrum in Fig. 2*a*. Therefore even drastic segregation of ice from a very dilute solution such as 10^{-3} M, resulting in a dramatic increase of the solute concentration, will not show up in the ESR spectrum. Figure 3*b* shows the ESR spectrum of a jet-frozen emulsion in the 'best' conditions for vitrification. These conditions were identical with the freezing conditions of the pure liquid water sample in Fig. 1*b*. The ESR spectrum is very similar to the spectrum of the perfectly vitrified solution in Fig. 2*a*. Some aggregation of CuCl_2 is indicated by a broad hump at 2,940 G.

The ESR spectra of jet-frozen 0.10 M CuCl_2 emulsions display drastic spectral changes with increasing temperature which are irreversible. In Fig. 4 the ESR spectra of a sample warmed for 30 min consecutively to 153 K (*b*), 173 K (*c*) and 193 K (*d*) have to be compared with the spectrum of the original sample at 113 K (*a*). At 153 K the ESR signal starts to broaden approaching within 30 min the width of a 1.5 M vitrified solution (compare with Fig. 2*b*). The increase of the signal at 2,940 G indicates the formation of a highly concentrated CuCl_2 phase.

At higher temperatures the amount of concentrated CuCl_2 increases. Evidence from DTA warm-up curves and from the X-ray diffraction patterns of Fig. 1a clearly indicates the process causing the irreversible change of the ESR spectra with temperature. The start at 153 K correlates well with the devitrification temperature determined by DTA. At this temperature ice I_c (shown by the X-ray diffraction pattern) starts to separate from the vitrified solution, the remaining CuCl_2 becoming more and more concentrated.

From the theory³ of the homogeneous nucleation of freezing, the cooling rate necessary for vitrifying a μm -sized droplet of water was estimated to be $>10^{10} \text{ K s}^{-1}$ and was considered to be unattainable in practice. To compare our technique with others and to obtain some idea of the relative effects of emulsification and of jet-freezing we investigated the freezing methods usually applied in freeze etching. These methods were too slow to give observable DTA devitrification peaks, and we therefore used ESR spectroscopy. Dropping of $1 \mu\text{l}$ droplets into liquid Freon at 120 K (the "standard freezing method"¹⁴—cooling rates up to 10^3 K s^{-1}), yields complete aggregation and the ESR spectrum of a frozen 0.10 M CuCl_2 solution is identical with the spectrum in Fig. 2c. Even the much faster cooling methods developed by Bachmann and Schmitt¹⁵ (spray-freezing and jet-freezing into liquid propane at 77 K, estimated cooling rates of 10^5 K s^{-1}) do not prevent segregation of the solute from the solvent. The ESR spectrum of a spray-frozen 0.10 M CuCl_2 solution in Fig. 3a shows mainly aggregated CuCl_2 and only a minor amount of less concentrated CuCl_2 is indicated by the shoulder at 3,150 G. Jet-freezing of a 0.10 M CuCl_2 solution through a $20 \mu\text{m}$ aperture with 10 atm working pressure into liquid propane gives similar results to spray-freezing. We also tried to obtain a comparison with the cooling methods applied in splat-cooling of liquid metals and alloys¹⁶ by jetting a 0.10 M CuCl_2 solution through a $10 \mu\text{m}$ aperture with 100 atm working pressure onto a copper plate cooled to 77 K. The jet was moved as quickly as possible over the surface of the cooled copper plate and its speed was calculated to be $\sim 100 \text{ ms}^{-1}$ from the diameter of the aperture and consumption of liquid. Although this experiment was not performed using strict splat-quenching conditions we

believe it to be comparable with the 'gun-technique'¹⁶ because of the high speed and small diameter of the jet. The ESR spectrum of the resulting frozen solid indicated complete aggregation. Apparently splat-cooling techniques give very low cooling rates for water and dilute aqueous solutions due to the low conductivities of water and ice.

We estimated our cooling rate by jet-freezing a non-emulsified 0.10 M CuCl_2 solution, using otherwise identical conditions (glass capillary with $50 \mu\text{m}$ aperture, 100 atm working pressure into liquid propane at 77 K). The ESR spectrum shows a clear improvement over the ESR spectrum of a spray-frozen sample in Fig. 3a, the signals at 3,050 and 3,150 G being of equal size. We therefore assume the cooling rate to be between 10^5 and 10^6 K s^{-1} . The further improvement with emulsified solutions in Fig. 3b demonstrates clearly the importance of emulsification and of the resulting supercooling effect for the vitrification of water and dilute aqueous solutions.

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- Burton, E. F. & Oliver, W. F. *Proc. R. Soc. A* **153**, 166 (1935).
- Venkatesh, C. G. & Rice, S. A. *Science* **186**, 927–928 (1974).
- Fletcher, N. H. *Rep. Progr. Phys.* **34**, 913–994 (1971).
- Pryde, J. A. & Jones, G. O. *Nature* **170**, 685–688 (1952).
- Angell, C. A. & Sare, E. J. *J. chem. Phys.* **49**, 4713–4714 (1968); **52**, 1058–1068 (1970).
- Wolstenholme, G. E. W. & O'Connor, M. *The Frozen Cell* (Churchill, London 1970).
- Rasmussen, D. H. & MacKenzie, A. P. *J. chem. Phys.* **59**, 5003–5013 (1973).
- McMillan, J. A. & Los, S. C. *Nature* **206**, 806–807 (1965).
- Rasmussen, D. & Luyet, B. *Biodynamica* **10**, 319–331 (1969).
- Luyet, B. & Rasmussen, D. *Biodynamica* **10**, 167–191 (1968).
- Dowell, L. G. & Rinfret, A. P. *Nature* **188**, 1144–1148 (1960).
- Olander, D. S. & Rice, S. A. *Proc. natn. Acad. Sci. U.S.A.* **69**, 98–100 (1972).
- Abraham, A. *The Principles of Nuclear Magnetism*, **126** (Clarendon, Oxford, 1973).
- Moor, H. & Mühliethaler, J. *J. Cell Biol.* **17**, 609–628 (1963).
- Bachmann, L. & Schmitt, W. W., *Proc. natn. Acad. Sci. U.S.A.* **68**, 2149–2152 (1971); in *Freeze Etching*, (eds Benedetti, & E. L. & Favard, P.) 73–79 (Soc. Franc. Microsc. Electron, Paris 1973).
- Jones, H. *Rep. Progr. Phys.* **36**, 1425–1497 (1973).

Lu–Hf total-rock isochron for the eucrite meteorites

P. J. Patchett*† & M. Tatsumoto*

* US Geological Survey, Denver Federal Center, Denver, Colorado 80225

† Colorado School of Mines, Golden, Colorado 80401

The isotope ^{176}Lu (2.6% of natural lutetium) decays by β^- to ^{176}Hf , with a long half life. We present here the first Lu–Hf isochron. The eucrite meteorites, a suite of planetary igneous rocks of known age, 4,550 Myr, define a 10-point total-rock isochron with a slope of 0.0934 ± 40 , leading to a value of $3.53 \pm 0.14 \times 10^{10} \text{ yr}$ for the β^- -decay half life of ^{176}Lu . The isochron intercept of 0.27973 ± 12 gives the initial $^{176}\text{Hf}/^{177}\text{Hf}$ for the inner Solar System at the time of accretion.

Previous geochronological use of the ^{176}Lu – ^{176}Hf decay scheme involved REE-rich minerals^{1,2} in attempts to determine the half life. Faure³ summarized all physical determinations and suggested a best estimate of $3.5 \pm 0.2 \times 10^{10} \text{ yr}$. However, because ^{176}Lu is an odd Z-odd N nuclide, then a branching decay is theoretically possible. Dixon *et al.*⁴ set an approximate upper limit of 3% on the electron capture to ^{176}Yb , but to date this possible decay has been neither clearly substantiated nor disproved. It has also not been clearly shown that the Lu–Hf method can be used in isotope chronology and geochemistry.

Using a wide variety of petrological and geochemical criteria, the eucrites constitute a coherent group of cumulate and noncumulate igneous rocks (brecciated to varying degrees), which may have come from a single-parent asteroid^{5–7}. In isotope geochemistry, the eucrites are important because they seem to have been produced during a major planetary differentiation 4,550 Myr ago, and to have been isotopically mainly

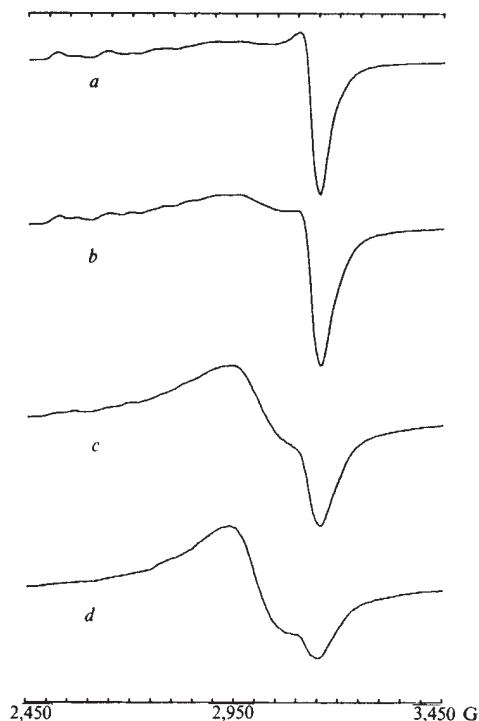


Fig. 4 Low temperature (113 K) ESR spectra of a jet-frozen 0.10 M aqueous CuCl_2/n -heptane emulsion: a, warmed at most to 113 K; b, c and d warmed consecutively for 30 min to 153 K, 173 K and 193 K respectively.

undisturbed since then. Extensive Rb–Sr (refs 8, 9), U–Pb (ref. 10), and Sm–Nd (refs 10–14) studies confirm this, and also that the samples had essentially the same $^{87}\text{Sr}/^{86}\text{Sr}$ and $^{143}\text{Nd}/^{144}\text{Nd}$ ratios 4,550 Myr ago. Thus although the eucrites may well have been derived from a single-parent body, this is not a necessary assumption for the present Lu–Hf total-rock isochron exercise, because they have already been shown to be isotopically concordant for the Rb–Sr and Sm–Nd systems, both in age and initial ratio.

Of the 10 samples studied, Stannern, ALHA 77302, Béréba, Nuevo Laredo, Pasamonte, Sioux County and Juvinas are non-cumulate eucrites, consisting of variously brecciated fine- to medium-grained basaltic material. Serra de Magé, Moore County and Moama are cumulate rocks⁶, consisting mainly of coarse-grained unbrecciated pyroxene and plagioclase.

The analytical method is described fully elsewhere¹⁵. Bomb dissolutions on the disaggregated meteorites were used. The samples were then separated into two aliquots, and mixed ^{176}Lu – ^{180}Hf spike added to one fraction. Chemical separation for Hf involved three ion-exchange columns and the overall chemical yield was 90%. Lu was not well separated from Yb, but the latter is more volatile and can be easily burned off the sample in the mass spectrometer. Overall blanks were <0.1 ng for Lu and Hf; even for the most Lu, Hf-poor sample, Moama, the sample/blank ratio is >600 for both elements, and hence no corrections for blanks were made. At most, this could result in a 0.15% bias in isochron slope; this is 30 times less than the 2σ slope uncertainty, and is clearly insignificant.

Lu was measured on Ta side filaments using a Re centre filament. Hf was measured using a triple Re filament assembly. $^{179}\text{Hf}/^{177}\text{Hf} = 0.7325$ was used for normalization. The mean value for 25 runs on the Johnson Matthey Hf standard JMC 475 is 0.282195 ± 15 (2σ) (see Fig. 1). We suggest JMC 475 as an international mass spectrometric Hf standard, and aliquots prepared from a single batch are available on request from Denver. From 1 μg of Hf, a total signal of 0.5×10^{-11} A can be maintained for over 3 h, leading to precision of 0.01–0.03% on $^{176}\text{Hf}/^{177}\text{Hf}$.

The low Hf contents of the cumulate eucrites Serra de Magé, Moore County and Moama necessitated use of ion counting for Hf composition runs on these samples. Our two-stage mass spectrometer is equipped with a multiplier as collector, and a pulse-height discriminator to detect pulses. JMC 475 was run using ion counting on the two-stage mass spectrometer immediately before each Hf run on the cumulate eucrites, because of possible bias introduced by using a different machine and by variations in multiplier peak shape. Adjusting the higher values measured for JMC 475 by counting to 0.282195 resulted in corrections of -0.00012 being applied to the Hf compositions of Moore County and Moama, and -0.00028 to Serra de Magé. Corrections for Yb interference in Hf runs were never necessary, and in normal samples, Lu was also absent for all but the first 10–30 ratios of a run. For the samples measured by count-

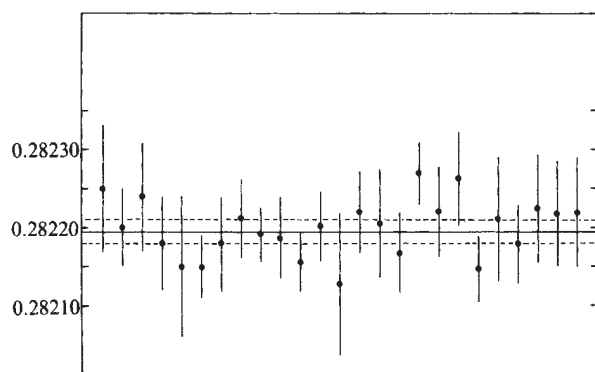


Fig. 1 $^{176}\text{Hf}/^{177}\text{Hf}$ ratios for the Johnson Matthey standard JMC 475 during the eucrite study. The overall mean is 0.282195 ± 15 normalized to $^{179}\text{Hf}/^{177}\text{Hf} = 0.7325$.

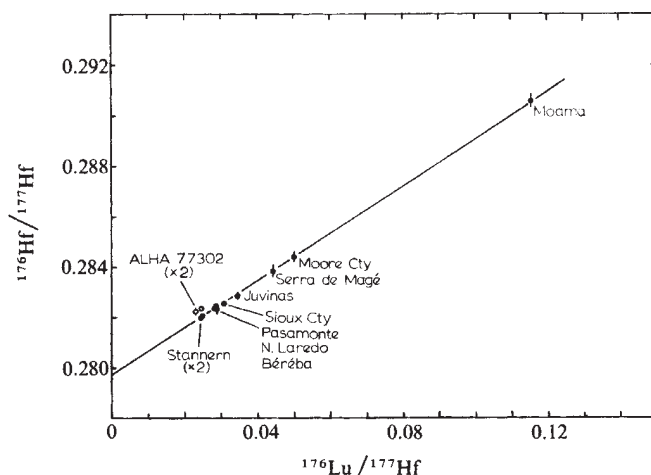


Fig. 2 Lu–Hf isochron for the eucrite meteorites. ALHA 77302 is excluded from the regression treatment. Slope, 0.0934 ± 0.0040 ; intercept, 0.27973 ± 0.00012 ; MSWD, 2.50.

ing, corrections for Lu were applied, amounting to absolute downward adjustments in $^{176}\text{Hf}/^{177}\text{Hf}$ of 0.00002–0.00008.

Lu–Hf isotopic results are listed in Table 1. The Lu/Hf ratio decreases with increasing Lu and Hf concentrations, so that the cumulate eucrites, depleted in trace elements, have Lu/Hf much higher than the noncumulate eucrites. However, over the Hf concentration range 2.4 p.p.m. (Stannern) to 0.8 p.p.m. (Juvinas), the Lu/Hf ratio changes only from 0.18 to 0.25, and there is thus strong coherence between the two elements. In the cumulates, where the residual liquid from crystallization of the pyroxene and plagioclase has been at least partly excluded, this coherence disappears. The cumulate samples (Moama, Lu/Hf = 0.82) presumably became more depleted in Hf than they did in Lu because Hf was more strongly concentrated in the residual liquid. This marked relative depletion of Hf in rocks which do not contain their lowest-melting components, such as cumulates, or residues of partial melting, seems to be an important characteristic of Lu–Hf geochemistry.

We have measured the isotopic composition of Lu in three samples, and find it to be within error of the terrestrial JMC 320 standard (37.701 ± 0.028), particularly taking into account the fact that fractionation cannot be normalized for this two-isotope element. Using large Lu^+ ion beams from triple filaments, and a Faraday cup collector, we consistently measure ~ 37.70 for JMC 320, and 37.55–37.74 for natural samples, depending on fractionation conditions. We cannot reproduce the low values of McCulloch *et al.*¹⁹, obtained using an electron multiplier, and because we can see no way to correct our measurements objectively for mass fractionation, we have internally standardized all our calculations by using the measured value of 37.701 ± 0.028 for natural $^{175}\text{Lu}/^{176}\text{Lu}$. If this ratio is used in any future Lu–Hf geochronology, then all errors will mutually cancel, because the decay constant determined here is based on an isochron whose slope is calculated using the same Lu isotopic composition. $^{180}\text{Hf}/^{177}\text{Hf} = 1.88651$ (normalized to $^{179}\text{Hf}/^{177}\text{Hf} = 0.7325$) was used for all natural Hf.

Except for the Antarctic eucrite ALHA 77302, the analyses define an isochron (Fig. 2). To increase precision on the initial $^{176}\text{Hf}/^{177}\text{Hf}$ value, both Stannern results have been included in the regression¹⁶. The MSWD value of 2.50 suggests a small degree of scatter beyond analytical uncertainties (see Fig. 3), and the 2σ errors in slope and intercept (Fig. 2) were obtained by multiplication of the *a priori* uncertainties with $\sqrt{\text{MSWD}}$ (ref. 16).

ALHA 77302 was analysed twice, and lies clearly above the isochron (Figs 2, 3). The sample was never homogenized, so that the two results are slightly different, with correlated Lu/Hf and $^{176}\text{Hf}/^{177}\text{Hf}$. ALHA 77302 has Lu and Hf concentrations very similar to Pasamonte, Nuevo Laredo and Béréba, and distinctly

Table 1 Lu-Hf isotopic data

	Lu(p.p.m.)	Hf(p.p.m.)	$^{175}\text{Lu}/^{176}\text{Lu}^*$	$^{176}\text{Lu}/^{177}\text{Hf}^\dagger$	$^{176}\text{Hf}/^{177}\text{Hf}^*$
Stannern	0.4112	2.3437	37.67 ± 9	0.024869 ± 59	0.282052 ± 43
Stannern	0.4145	2.3969	—	0.024510 ± 59	0.281953 ± 78
ALHA 77302	0.2732	1.5438	—	0.025083 ± 60	0.282418 ± 58
ALHA 77302	0.2599	1.5387	—	0.023937 ± 57	0.282316 ± 144
Nuevo Laredo	0.3599	1.8125	—	0.028142 ± 67	0.282372 ± 66
Béréba	0.2649	1.2968	—	0.028957 ± 69	0.282322 ± 170
Pasamonte	0.2984	1.4792	37.74 ± 3	0.028593 ± 67	0.282490 ± 63
Sioux County	0.2439	1.1165	—	0.030965 ± 92	0.282543 ± 94
Juvinas	0.1946	0.7991	—	0.034530 ± 81	0.282848 ± 109
Serra de Magé	0.04297	0.1374	—	0.044342 ± 135	0.283800 ± 209‡
Moore County	0.1823	0.5159	37.62 ± 15	0.050105 ± 152	0.284384 ± 202‡
Moama	0.06631	0.08136	—	0.11571 ± 33	0.290587 ± 253‡

* In-run errors are $2\sigma_m$.

† Error includes in-run uncertainties from spiked Lu and Hf runs, an estimated 0.04% uncertainty on run-to-run Lu mass fractionation, and uncertainties on Lu and Hf spike concentration.

‡ Hf composition measured using ion counting on a different mass spectrometer. As a result of standards measured at the same time, Serra de Magé Hf composition has been corrected by -0.00028, and Moore County and Moama by -0.00012 to correspond to the other results.

lower than Stannern (Table 1). The regular decrease of Lu and Hf upwards along the isochron suggests that the Hf isotopic composition of ALHA 77302 is correct and original (that is similar to Pasamonte, Nuevo Laredo and Béréba), but that the Lu/Hf ratio has been disturbed in post-crystallization time. It seems possible that ALHA 77302 has lost Lu in the recent past, perhaps while embedded for a long time period in the Antarctic ice. The only alternative explanation, that the Hf isotopic composition has been contaminated, is unlikely because any Hf available in quantity on the Earth is probably no more radiogenic than $^{176}\text{Hf}/^{177}\text{Hf} = 0.284$ (unpublished data), so that a large proportion of such Hf would be needed to raise ALHA 77302 above the isochron. However, we have not conducted leaching experiments on this eucrite.

The intercept of the isochron, 0.27973 ± 12 , gives the $^{176}\text{Hf}/^{177}\text{Hf}$ of the eucrites at 4.550 Myr, assuming that the age established by other methods can be used for Lu-Hf. As in the case of Nd, it is reasonable, in the absence of evidence to the contrary, to use this value as the primordial Hf isotopic composition for the planets of at least the inner Solar System. At present, however, the chondritic and bulk Earth Lu/Hf ratios are very poorly known, so that isotopic evolution lines cannot be drawn accurately until more data become available. This lack of knowledge is the result of Lu having been analysed with other REE, and Hf usually separately in studies of Zr-Hf fractionation. Our duplicated Hf concentration for Stannern (2.36 p.p.m.) disagrees not only with the neutron activation data of Ehmann *et al.*¹⁷ (1.26 p.p.m.), but also with the isotope

dilution results of Shima¹⁸ (1.23 p.p.m.). This discrepancy can probably only be accounted for by sample heterogeneity, but if our Hf contents of Pasamonte and Stannern are combined with the Zr data of Ehmann *et al.*¹⁷, then normal Zr/Hf ratios of 35 and 40 are obtained, in contrast to the anomalous 52 and 75 which these authors find using their own Hf contents.

The slope of the isochron, 0.0934 ± 0.0040 , can be used to calculate the β^- -decay constant of ^{176}Lu , assuming that the total-rock eucrites have been isotopically undisturbed since 4,550 Myr (refs 7-13). The following values are obtained:

$$\lambda^{176}\text{Lu}(\beta^-) = 1.962 \pm 0.081 \times 10^{-11} \text{ yr}^{-1}$$

$$t_{1/2}^{176}\text{Lu}(\beta^-) = 3.53 \pm 0.14 \times 10^{10} \text{ yr}$$

These 2σ errors include the scatter component on the isochron; if only the *a priori* uncertainty¹⁶ is used, then the uncertainties on λ and $t_{1/2}$ are reduced to 0.051 and 0.081 respectively.

The weighted mean of five physical half life determinations made since 1960 (summarized in ref. 3) is $3.54 \pm 0.11 \times 10^{10}$ yr; this is unchanged if the earlier geological determination² of $3.3 \pm 0.5 \times 10^{10}$ yr is included. The agreement between the mean of the physical determinations and the present value based on a Lu-Hf isochron is impressive, and suggests that the value 3.53×10^{10} yr for the half life (corresponding to $\lambda = 1.96 \times 10^{-11} \text{ yr}^{-1}$) can now be adopted.

We have ignored the possibility of a minor electron-capture decay of ^{176}Lu to ^{176}Yb , so that our half life may be slightly too long. Even if the electron capture does occur, any errors in the age determination will be cancelled out if the half life based on the eucrite isochron is used in future work. This is because the percentage effect on age of an unaccounted-for minor branching decay is essentially linear with time when the decay is slow, as for ^{176}Lu . If the electron-capture decay can be confirmed and its proportion fixed, then ages calculated using the half life determined here will not need adjustment.

We have established here the usefulness of the ^{176}Lu - ^{176}Hf decay scheme in isotope chronology, and shown that the igneous eucrite meteorites define an isochron, the slope of which allows the β^- -decay constant of ^{176}Lu to be determined. The initial $^{176}\text{Hf}/^{177}\text{Hf}$ ratio of the eucrites at 4,550 Myr probably applies to the Solar System generally, and will be of great importance in calculations of the isotopic evolution of planetary bodies and meteorites.

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- Herr, W., Merz, E., Eberhardt, P. & Signer, P. *Z. Naturf.* **13a**, 268-273 (1958).
- Boudin, A. & Deutsch, S. *Science* **168**, 1219-1220 (1970).
- Faure, G. *Principles of Isotope Geology* (Wiley, New York, 1977).

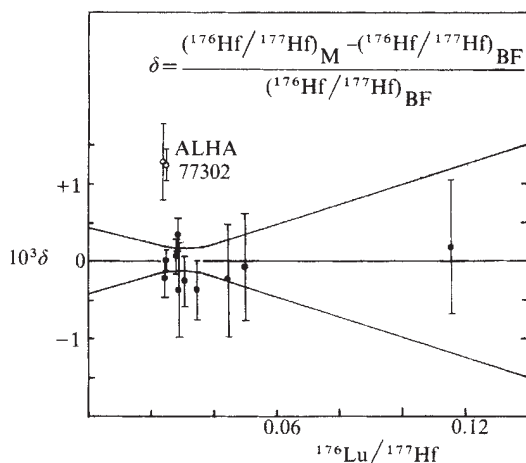


Fig. 3 Deviations in measured $^{176}\text{Hf}/^{177}\text{Hf}$ (M) of the analyses from those predicted by the best fit line (BF). Isochron error envelope and error bars are 2σ . As in the isochron diagram, ALHA 77302 lies clearly above the line.

4. Dixon, D., McNair, A. & Curran, S. C. *Phil. Mag.* **45**, 683–684 (1954).
5. Duke, M. B. & Silver, L. T. *Geochim. cosmochim. Acta* **31**, 1637–1665 (1967).
6. Stolper, E. *Geochim. cosmochim. Acta* **41**, 587–611 (1977).
7. Consolmagno, G. J. & Drake, M. J. *Geochim. cosmochim. Acta* **41**, 1271–1282 (1977).
8. Papanastassiou, D. A. & Wasserburg, G. J. *Earth planet. Sci. Lett.* **5**, 361–376 (1969).
9. Birck, J. L. & Allègre, C. J. *Earth planet. Sci. Lett.* **39**, 37–51 (1978).
10. Unruh, D. M., Nakamura, N. & Tatsumoto, M. *Earth planet. Sci. Lett.* **37**, 1–12 (1977).
11. Lugmair, G. W. *Meteoritics* **9**, 369 (1974).
12. Nakamura, N., Unruh, D. M., Gensho, R. & Tatsumoto, M. *Lunar Sci. VIII Abstr.* 712–713 (1977).
13. Lugmair, G. W., Scheinin, N. B. & Carlson, R. W. *Meteoritics* **12**, 300–301 (1977).
14. Hamet, J., Nakamura, N., Unruh, D. M. & Tatsumoto, M. *Proc. 9th Lunar Sci. Conf.* 1115–1136 (1978).
15. Patchett, P. J. & Tatsumoto, M. *Contr. Miner. Petrol.* (in the press).
16. York, D. *Earth planet. Sci. Lett.* **5**, 320–324 (1969).
17. Ehmann, W. D., Chyi, L. L., Garg, A. N. & Ali, M. Z. in *Origin and Distribution of the Elements* Vol. II (ed. Ahrens, L. H.) 247–259 (Pergamon, Oxford, 1977).
18. Shima, M. *Geochim. cosmochim. Acta* **43**, 353–362 (1979).
19. McCulloch, M. T., DeLaeter, J. R. & Rosman, K. J. R. *Earth planet. Sci. Lett.* **28**, 308–322 (1976).

Is the Hercynian belt of Brittany a major shear zone?

Denis Gapsis & Claude Le Corre

Centre Armoricain d'Etude Structurale des Socles (CNRS),
Université de Rennes, avenue du Général-Leclerc, 35042 Rennes
Cédex, France

In the Hercynian belt of Brittany (France), large localized shear zones indicate dextral transcurrent shearing. In this letter regional Hercynian structures and their relationships with shearing are considered and information obtained by quartz fabric analysis in the central part of the belt, outside the major shear zones, is presented. Fabric patterns show that a dextral shear component is associated with Hercynian penetrative deformation. Existing geological data are reconsidered in the light of these results, and the extent to which the Hercynian belt of Brittany can be regarded as a 'major shear zone' is discussed.

The Hercynian belt of Brittany comprises different structural domains which are separated by major crustal discontinuities¹ expressed as transcurrent shear zones (Fig. 1). Within this segment of the west-European Hercynides, Central Brittany is an essentially Hercynian domain^{2,3} characterized by structures which follow a roughly east–west trend (Fig. 1). In Central Brittany tectonic structures show the effects of (1) a roughly north–south shortening, (2) diapiric ascent of Hercynian granites, and (3) a dextral transcurrent shearing. Two basic geodynamic models, which involve mainly large-scale transcurrent shearing⁴ or north–south compression¹, have been proposed to account for Hercynian deformation in Central Brittany. In fact, the problem of the bulk deformation regime (coaxial or non-coaxial) is not at all clear. Recent studies have shown that, when developed during shearing, crystallographic fabrics of quartz are generally asymmetric and can be used to deduce the sense of shearing (see ref. 5). Perhaps the first clear field indication of a close relationship between dextral shearing and Hercynian deformation in Central Brittany was that reported by Bouchez & Blaise⁶ and Bouchez⁷, on the basis of quartz fabric data. Their results, however, are of very local applicability. Furthermore, clear microtectonic evidence of shearing is lacking within large parts of the belt^{8–10}. This has led to a modified geodynamic model⁹ in which effects of shearing are mainly restricted to the mapped shear zones (Fig. 1), and remain relatively minor compared with a north–south compression.

Central Brittany is bounded to the north by a rigid continental block, which has been stable since the end of the Proterozoic (see, for example, Mancelian granites) and only slightly affected by the Hercynian orogeny. To the south lies a crystalline block affected mainly by Siluro–Devonian tectono-metamorphic events¹. Both the northern and the southern limits of Central Brittany are defined by major ductile fault zones, the North and South Armoricain shear zones^{1,12–16}. These shear zones, which have a long and complex structural history^{1,15,16}, have acted as dextral strike-slip fault zones up to late Hercynian

times^{14,15,17,18}. Hercynian plutonism^{19,20} is concentrated along these shear zones (Fig. 1).

Within Central Brittany, deformation of the sedimentary rock (Proterozoic and Palaeozoic) is mainly expressed as a regional cleavage, generally subvertical, and associated with large-scale folding. Folds are generally upright with low amplitudes^{9,10,21}. Fold axes are always subhorizontal or gently plunging. The cleavage is parallel to the principal plane (xy) of the finite strain ellipsoid, and generally exhibits a mineral elongation lineation parallel to the x axis of the finite strain ellipsoid^{8,22}. This principal extension direction is parallel, or nearly so, to the regional fold axes. The regional trend of folds and cleavage trajectories varies from west–southwest to east–southeast when moving eastwards through the belt³. Local data relating to finite strain (refs 7–9, 11, 22, and S. K. Hanmer, personal communication) indicate a large range of possible shapes of the finite strain ellipsoid. However, when present, stretching on the intermediate axis (y) remains generally weak or moderate. Plane strain ellipsoids are common and even constrictive finite strain is observed.

The axial region of Central Brittany appears as a zone of relatively weak deformation. Zones of more intense deformation are found on moving north or south, towards the North and South Armoricain shear zones. Thus, across the area between Rennes and the southern branch of the South Armoricain shear zone (Fig. 1), we have shown that strain intensity increases towards the south, as a result of Hercynian deformation^{8,9,11}. We have also shown^{8,9,11,23} that this regional strain gradient is associated with an increase of metamorphism towards the south³.

The Hercynian granites were demonstrably intruded during penetrative deformation^{2,3,11}. Regional patterns of strain and metamorphism change in the vicinity of plutons (see Fig. 2). Local perturbations can also be observed around pre-Hercynian bodies (for example, Mancelian granites), as a result of their rigid behaviour relative to the country-rocks during the Hercynian orogeny.

Fabrics and optical microstructures of quartz have been studied in detail within a quartzitic series (Armorican quartzite) of lower Ordovician age. Results given in full elsewhere¹⁵

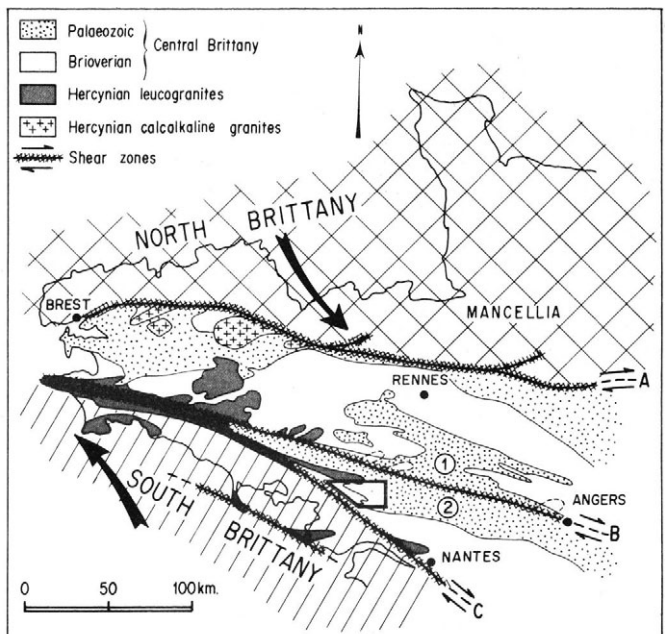
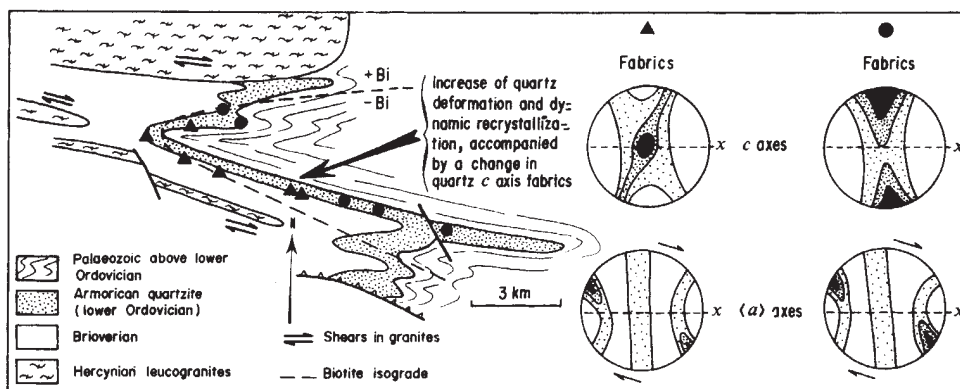


Fig. 1 Simplified geological map of Brittany. The large-scale movements are not precisely determined and arrows indicate only a regional dextral shear component. ①, St Georges-sur-Loire Synclinorium (the inset corresponds to the area of Fig. 2); ②, Sud de Rennes Synclines; A, North Armoricain shear zone; B + C, South Armoricain shear zone (B, northern branch; C, southern branch).

Fig. 2 Geological map of the western part of the St Georges-sur-Loire Synclinorium and variations of fabrics and optical microstructures of quartz within the Armorican quartzite. The dotted line indicates the approximate biotite isograd. The schematic fabric diagrams represent the characteristic c axis and (a) axes patterns, as inferred from detailed texture goniometry study^{10,11}. These patterns indicate a dextral shear component. In the field, the cleavage (dotted line on pole figures, foliation in quartzites) is subvertical, trends roughly N 110°, axial-plane of regional folds. The shear component is acting within a sub-vertical plane, along a subhorizontal direction.



were obtained from the western part of the St-Georges-sur-Loire Synclinorium (Figs 1 and 2). This Hercynian structure is bounded to the west by Hercynian leucogranites. Associated thermal anomalies are revealed by biotite isograds (Fig. 2). Moving westwards along the southern limb of the synclinorium, an increase of intracrystalline deformation and dynamic recrystallization is observed, leading to a fine-grained quartz mylonite. This is accompanied by a change in quartz c -axis fabric from a broad girdle with a maximum near the axis of principal shortening to a crossed-girdle pattern with a maximum near the y axis of the finite strain ellipsoid (Fig. 2). This deformation gradient is attributed to the thermal softening induced by the granites¹¹. Quartz fabrics are typically monoclinic. They show a strong maximum of (a) axes oblique with respect to the principal stretching direction x , and also unequally populated girdles of c -axes. Such fabrics indicate a dextral shear component^{5,10,24} within a subvertical plane, irrespective of the microstructures exhibited by the quartzites (Fig. 2). In some of the quartzites, the occurrence of shear bands and of sigmoidal shadow-zones around rigid objects give further evidence of dextral shearing¹⁰. Our observations infer that regional shortening and emplacement of granites took place during shear deformation. The leucogranites are themselves penetratively sheared and stretched along the regional extension direction (Figs 1 and 2). Further south from the St-George-sur-Loire Synclinorium, the South Armorican shear zone (Fig. 1) is evidence for more intense shear. On the other hand, on moving north, within moderately deformed zones (such as the Sud de Rennes synclines, Fig. 1), the influence of a shear component becomes less apparent. Over large areas, we have not found any clear evidence for shearing during the main deformation, either from microtectonic criteria or fabric data⁸⁻¹⁰. Indeed, within this domain, the only clear evidences of shearing that has ever been reported is along the northern branch of the South Armorican shear zone^{6,17,18} and also along discrete strike-slip microfaults in slates of the country-rocks⁹.

The overall geometry of the structures in Central Brittany (general trends of fold axes, cleavage trajectories and mapped shear zones; principal extension direction subhorizontal and parallel to fold axes) suggests the existence of large-scale dextral transcurrent shearing^{4,6}. As shown above, regional strain gradients seem to be closely related to variations in intensity of shearing. Other evidence indicates that the ductile fault zones have acted as dextral strike-slip fault zones, not only in late Hercynian times, but also during the main Hercynian deformation, with associated local strain and metamorphism gradients^{6,14}.

Fabric analysis and microtectonic analysis have not given any unequivocal evidence of non-coaxial deformation in weakly deformed zones. This kind of approach, though quite useful in sufficiently deformed rocks, seems to be less satisfactory for rocks exhibiting low strain intensities, where a stable fabric is not attained. Such investigations, if restricted to moderately deformed zones, can therefore lead to incorrect assumptions about the bulk deformation history. Thus, in the area under consideration, it gives only further evidence for regional short-

ening. Also, finite strain assessments which are locally significant cannot be directly extrapolated to provide a quantitative estimate of the geometry of the bulk strain (see ref. 25). In Central Brittany, perturbations induced by granitic bodies introduce further complications. Nevertheless, the overall low amplification of regional folds, bearing in mind that significant ductility contrasts exist between folded series^{9,10,21}, is consistent with the observed principal extension subhorizontal and parallel to fold axes and with no evidence for important vertical finite extension. This pattern can be compared with results of bulk plane strain folding experiments²⁶. In this way, deformation by shearing is thought to be the main factor limiting the vertical finite extension in Central Brittany.

An oblique convergence of a northern and a southern continental block might account for the observations and would be consistent with structural correlation that can be made between South and Central Brittany²⁷. However, it remains difficult to estimate to what extent the bulk process may have differed from a regional simple shearing model. This might be overcome by extending detailed finite strain analysis throughout the whole region to complete the information about strain gradients and be able to calculate the effects of removing strains^{28,29}. It has been suggested that the granites along the major shear zones could be the result of localized crustal melting by shear heating^{6,30}. However, it has been thought that shear heating cannot in general be sufficient for this purpose³¹. Furthermore, the pattern of regional metamorphism is associated with a regional thermal anomaly which can account for the Hercynian plutonism³. The relation between major shear zones and granites can be explained by a thermally initiated strain softening in these zones³². Before the shear zones get sufficiently soft to allow most of the deformation to occur in these zones, it is reasonable to expect the entire region to deform as a whole. Regional Hercynian structures could, at least in part, be interpreted in this way. Then, dextral movements could continue along the main shear zones, up to late Hercynian times.

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1. Cogné, J. in *Colloq. int. CNRS, Géologie de l'Himalaya*, **168**, 111-129 (1977).
2. Le Corre, Cl. *Bull. B.R.G.M.* **3**, 219-254 (1977).
3. Hamner, S. K., Le Corre, Cl. & Berthé, D. *J. Geol. Soc. London* (in the press).
4. Matte, Ph. & Ribeiro, A. *C. R. heb. Séanc. Acad. Sci., Paris D280*, 2825-2828 (1975).
5. Bouchez, J. L. *Tectonophysics* **49**, T25-T30 (1978).
6. Bouchez, J. L. & Blaise, J. *Bull. Soc. géol. Fr.* **1**, 145-157 (1976).
7. Bouchez, J. L. *Tectonophysics* **39**, 25-50 (1977).
8. Le Corre, Cl. & Le Théoff, B. *Bull. Soc. géol. Fr.* **6**, 1435-1442 (1976).
9. Le Corre, Cl. thesis, Univ. Rennes (1978).
10. Gapais, D. thesis, Univ. Rennes (1979).
11. Gapais, D. *Bull. Miner.* **102**, 249-264 (1979).
12. Chauris, L. *C. R. heb. Séanc. Acad. Sci., Paris D268*, 2859-2861 (1969).
13. Paris, F. & Jegouzo, P. *4ème Réunion. Ann. Sci. Terre*, Paris (1975).
14. Hirbec, Y. thesis, Univ. Rennes (1979).
15. Watts, M. J. & Williams, G. D. *J. struct. Geol.* **1**, 323-332 (1979).
16. Jegouzo, P. *J. struct. Geol.* **2**, 39-47 (1980).
17. Berthé, D., Choukroune, P. & Jegouzo, P. *J. struct. Geol.* **1**, 31-42 (1979).
18. Berthé, D., Choukroune, P. & Gapais, D. *Bull. Miner.* **102**, 265-272 (1979).
19. Vidal, Ph. *Bull. Soc. géol. Fr.* **15**, 232-245 (1973).
20. Peucat, J. J., Charlot, R., Mifdal, A., Chantreine, J. & Autran, A. *8ème Réunion. Ann. Sci. Terre*, Marseille (1980).
21. Pivette, B. thesis, Univ. Rennes (1978).
22. Claffich, M. *Bull. Soc. géol. Fr.* **3**, 757-762 (1976).

23. Le Corre, Cl. *Bull. Soc. géol. Fr.* **4**, 547–553 (1975).
24. Lister, G. S. & Williams, P. F. *J. struct. Geol.* **1**, 283–297 (1979).
25. Cobbold, P. *Can. J. Earth Sci.* **8**, 1721–1731 (1977).
26. Watkinson, A. J. *Tectonophysics* **28**, T7–T11 (1975).
27. Vigneress, J. L. *Soc. Géol. minér. Bretagne* (in the press).
28. Cobbold, P. *J. struct. Geol.* **1**, 67–72 (1979).
29. Percevault, M. N. & Cobbold, P. *Séme Réunion. Ann. Sci. Terre, Marseille* (1980).
30. Nicolas, A., Bouchez, J. L., Blaise, J. & Poirier, J. P. *Tectonophysics* **42**, 55–73 (1977).
31. Brun, J. P. & Cobbold, P. *J. struct. Geol.* **2**, 149–158 (1980).
32. Poirier, J. P., Bouchez, J. L. & Jonas, J. J. *Earth planet. Sci. Lett.* **43**, 441–453 (1979).

Clays from New Zealand support the inactive particle theory of soil sensitivity

I. J. Smalley, Craig W. Ross & J. S. Whitton

Soil Bureau, Department of Scientific and Industrial Research, Lower Hutt, New Zealand

As part of a long-term study of factors contributing to soil sensitivity, investigations have been carried out on some volcanic ash soils from the North Island of New Zealand. We report here that some soils have sensitivities of up to 140 and clay mineral contents of >80%. They apparently owe their high sensitivity to the fact that the clay is halloysite of the spherical variety, and that interparticle interactions with this particular soil mineral are remarkably slight. Although the clay soil has a high liquid limit (~65–80), it has a low plasticity index (~15–30) and its behaviour supports the inactive particle theory of soil sensitivity. When plotted on the Casagrande Plasticity Chart all samples plotted well below the 'A' line.

Sensitivity is a measure of the loss in strength which occurs when a soil is disturbed or remoulded. In extreme cases the undisturbed soil strength can be several hundred times as great as the remoulded strength². The ratio of undisturbed to remoulded strength indicates the sensitivity (S_r). Soils of extremely high sensitivity (sometimes called quickclays; $S_r > 50$) exist in eastern Canada and in Scandinavia and cause problems by landsliding and retrogressive flowslide behaviour. Various theories have been advanced to explain their properties: the role of interparticle cementation³, the leaching theory which has now evolved into a generalized electrochemical approach⁴ and the inactive-particle, short-range-bond theory¹⁵ are all being discussed. But choosing between the various approaches has been difficult because of the limited number of high sensitivity soils available. This situation has been helped by the discovery of soils with sensitivities reaching 140 in New Zealand. Initial investigations suggest that the cementation and Rosenqvist factors may not apply.

In August 1979 a damaging landslide occurred at Tauranga, on the northeastern fringe of the central volcanic plateau in the North Island of New Zealand. This landslide had many of the characteristics of a flowslide which occurs in highly sensitive soils and sensitivities of up to 140 were measured, although many much lower values were also recorded (C. Gulliver, personal communication). Mineralogical analyses by differential thermal analysis and X-ray diffraction (XRD) showed that the soil was largely composed (80%+) of hydrated halloysite (10 Å spacing on XRD); minor amounts of quartz and cristobalite were observed. At first sight this apparently contradicts the major requirement of the inactive particle theory, that the clay mineral content be small to negligible; however, the nature of the Tauranga halloysite is such that the observations and the requirements of theory can be reconciled. The inactive particle theory explains very high sensitivities by requiring that there be very little long-range attraction between the soil particles. In the Northern Hemisphere, this requirement is met by specifying that there should be very little active clay mineral content in the soil, and a recent programme of analyses on Canadian soils has shown that they do have low clay mineral contents^{6–8}. But in New Zealand it is possible to find soils with a very high clay

mineral content which do not have the 'clayey' or high plasticity indices of soils of similar clay proportion encountered in Northern Hemisphere engineering soil investigations.

Scanning electron microscope studies (by G. Walker; see Fig. 1) show that the soil at Tauranga is composed essentially of spherical halloysite particles. The analytical results from scanning electron microscopy/electron diffraction by X-ray analysis (SEM/EDAX) show a $\text{SiO}_2/\text{Al}_2\text{O}_3$ ratio of 1:1, which supports halloysite. Some small concretions were completely localized concentrations of iron and manganese (in roughly equal proportions); no sign of cementation was detected. These spherical clay particles (which are single crystalline units and not aggregations) form a very inactive clay with very small interaction with both positive and negative soil water ions; the phosphate uptake for a similar soil is the lowest measured in New Zealand⁹. Thus, although clay mineral particles predominate in the Tauranga material, long-range interparticle forces are essentially absent and the sensitivity is relatively high. The inactive particle theory will need to be generalized to require an absence of long range forces in the soil system rather than a lack of clay mineral materials, although investigations on a sensitive Norwegian soil¹¹ show that the minimum clay mineral requirement may still operate in the Northern Hemisphere.

Some structural collapse must be involved in the classic quickclay failure and yet the spherical particles might be expected to form a fairly compact structure. SEM reveals some variation in density with 'pores' distributed throughout the soil, and these seem to cause fairly low bulk densities, usually in the range 1.5–1.6 tonnes m^{-3} . Natural water contents of the sensitive ash soils were exceptionally high (60–100%) and were often above the liquid limits. The abundant water supports the separated particle once failure has occurred⁵ but the initial brittle failure, leading to structural collapse, occurs because of a lack of long-range bonds in the system. Handy¹¹ has shown that structural collapse in the loess soils of Iowa is promoted by an absence of clay minerals and this observation could indirectly support the inactive particle sensitivity theory. Fedá¹² and Denisov¹³ regard loess as a sensitive soil and relate its structural collapse on wetting to the total structural collapse exhibited by a quickclay when it is remoulded. In Iowan loess the primary mineral particles which form the basis of the soil structure are usually quartz with diameters of 20–60 μm , in a Canadian quickclay the particles are quartz and feldspar and the diameters are around 2–5 μm and in the New Zealand volcanic soil the

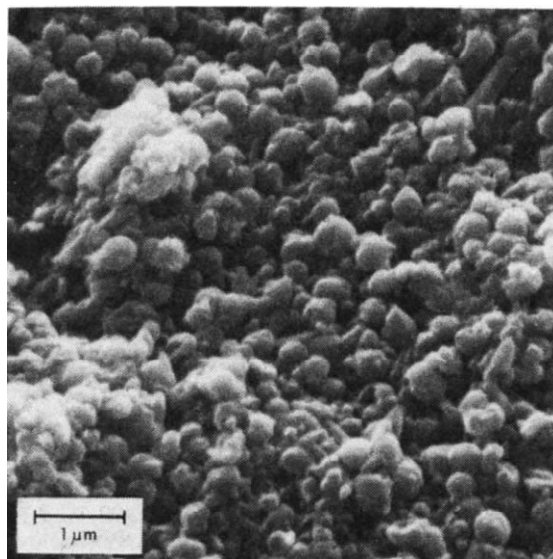


Fig. 1 Spherical halloysite particles comprising the sensitive volcanic ash soil at Tauranga; each particle has a diameter of ~0.2 μm . The particles are single crystalline units and not aggregates of smaller particles. (Photo: G. D. Walker, DSIR New Zealand.)

major soil particles are halloysite spheroids with diameters of $\sim 0.2 \mu\text{m}$; they all seem to have a propensity for structural failure, and they all lack long-range forces. (But we have a few reservations about considering loess a truly sensitive soil.)

The existence of the Tauranga soil, with a sensitivity that puts it in the quickclay class (using the Swedish definition) and a remarkable mineralogy, offers the chance of taking another small step towards a general theory of sensitivity. It also supports the approach suggested in the inactive-particle, long-range-bond theory, and it might offer some common ground on which to reconcile the revised approach with the generalized Rosenqvist theory.

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1. Smalley, I. J. *Nature* **231**, 310 (1971).
2. Penner, E. *Nature* **197**, 347–348 (1963).
3. Yong, R. N., Sethi, A. J., Booy, E. & Dascal, O. *Engng Geol.* **14**, 83–107 (1979).
4. Rosenqvist, I. Th. *Geol. Appl. Idrogeol.* **10**, 21–32 (1975).
5. Cabrera, J. G. & Smalley, I. J. *Engng Geol.* **7**, 115–133 (1973).
6. Smalley, I. J., Bentley, S. P. & Moon, C. F. *Can. Miner.* **13**, 364–369 (1975).
7. Bentley, S. P. & Smalley, I. J. *Can. Miner.* **16**, 103–112 (1978).
8. Bentley, S. P. & Smalley, I. J. *Engng Geol.* **14**, 209–217 (1979).
9. Theng, B. K. G., Russell, M., Churchman, G. J. & Parfitt, R. L. *Clays Clay Miner.* (in press).
10. Bentley, S. P., Clarke, N. J. & Smalley, I. J. *Can. Miner.* (in the press).
11. Handy, R. L. *Proc. Soil Sci. Soc. Am.* **37**, 281–284 (1973).
12. Feda, J. *Engng Geol.* **1**, 201–219 (1966).
13. Denisov, N. Ya. *Osnov. Fundam. Mekh. Grunt.* **5**, 5–8 (1963).

Formation of C_4 – C_7 hydrocarbons from bacterial degradation of naturally occurring terpenoids

John M. Hunt, Robert J. Miller & Jean K. Whelan

Woods Hole Oceanographic Institution, Woods Hole, Massachusetts 02543

We have recently found many light hydrocarbons, both alkanes and alkenes, in trace amounts (ng compound per g sediment) in Recent marine sediments^{1–4}. These hydrocarbons are believed to originate from both biological and low-temperature reactions in the sediments. Understanding their mechanism of formation may allow use of these compounds to decipher the past biological and thermal history of the sediments⁴. To investigate biological origins, we have cultivated mixed populations of bacteria on natural terpenoids and found, as degradation products, both alkanes and alkenes in the C_1 – C_7 range; this is the first report of C_4 – C_7 hydrocarbons being formed from microbial activities. Aerobic followed by anaerobic degradation yielded mainly small amounts of straight-chain alkenes. No such products resulted from blanks or controls. The results are consistent with products observed in natural environments.

The analytical procedure was carried out in 125-ml glass bottles (Hungate method)⁵ and 455-ml cans. Both types of vessels were equipped with silicone rubber septa² and shown to be gas tight by injecting a positive pressure of helium and testing with an electronic helium leak detector (Gow Mac).

All gases were passed through an alumina trap immersed in dry ice and an Oxisorb trap (Analabs) to remove traces of hydrocarbon or oxygen. All distilled water used in making solutions and media was sparged with helium for 5 min to remove any traces of volatile hydrocarbons. All anaerobic operations and transfers were carried out in a glove bag (12 R) which was alternately evacuated and filled three times with a mixture of 80% N_2 –20% CO_2 (the CO_2 was Matheson, Anaerobe purity). Vessels were kept in the glove bag maintained with a slight positive pressure of 80% N_2 –20% CO_2 during bacterial growth to guard against accidental small leaks.

Media was artificial seawater⁶ and other nutrients including Pfennig's⁷ trace metal stock solution (1 ml per litre). About 30 ml of this media (sterilized and sparged with 20% CO_2 –80% N_2) containing 0.5–1.5 mg terpene in suspension (farnesol or β -

carotene) per ml medium were added to the sterilized cans and the cans sealed.

A mixed culture of anaerobic bacteria was collected from the sediments in a brackish water pond (Salt Pond) near our laboratory in a collection tube, which was evacuated and filled with an atmosphere of 80% N_2 –20% CO_2 . The sample was brought back to the laboratory and further operations were carried out in the glove bag described above. The material was filtered through a 1.2- μm Millipore filter to exclude particulate matter and organisms larger than the desired bacteria. Portions of the filtrate (1 ml) were inoculated into sample cans containing media and farnesol or β -carotene as described above and blank cans which contained everything but the terpene (farnesol or β -carotene). Additional blank cans which had media and terpene substrates but no bacteria were also run with the other blanks. The cans were stored at room temperature and analysed as described below at the end of 144 h.

In the second method, the media was prepared in a device similar to that of Willingham⁷. The media (35 ml) (artificial seawater/Pfennig's solution as described previously plus 0.5 g per litre cysteine-HCl and a few drops of reazurin indicator solution dispensed into the serum bottles after CO_2 was bubbled through the dispensing device so that the indicator turned from blue to red) was dispensed into the bottles which were subsequently sealed. Substrates and bacteria were added in the same concentrations as used in the cans. The bottles were kept at room temperature and analysed after 196 h. During this time, the reazurin indicator turned from pink to colourless indicating anoxic conditions. Several blanks were run, some using substrates and all carbon sources but no bacteria, others using bacteria and media as described above, but no terpene.

We also wanted to know whether aerobic bacterial metabolism could modify functional groups and make them more susceptible to anaerobic attack. About 4 g of a Noble bacto-agar (VWR) was dissolved by heating in 1 l of a mineral salts medium, autoclaved, cooled and divided into portions. Carbon sources (1.5 g yeast extract and 1.5 g tryptose per litre of medium) were added and several plates were filled with 20 ml of medium. The terpenoids (farnesol and β -carotene) were added initially in low concentrations (10% terpenoid carbon source, 90% yeast/tryptose) and concentrations were gradually increased to prevent inhibition of bacterial growth from too much terpenoid. The final carbon source was 90% terpenoid and 10% yeast extract/tryptose. High bacterial populations were observed by direct microscopic examination for 7 days at these concentrations. Culturing was terminated by dissolving the agar from each plate with concentrated (12 M) HCl (15 ml) and neutralizing with NaOH to pH 7. This solution was placed in the glove bag, sparged with 80% N_2 –20% CO_2 , and 30 ml added to the 125-ml Hungate bottles along with 20 ml of the reduced media described above. The bottles were sealed and anaerobic sediment inoculum were introduced into the samples as described previously. Blanks were run with everything but the bacteria and with everything but the terpenoids. Samples were maintained at room temperature and analysed after 210 h. Cultures were terminated by injecting 5 ml of 0.1 M HgCl_2 into each bottle.

To drive all volatile compounds into the gas phase, the containers were heated at the end of the experiment in a hot water bath at 90°C for 15 min. An aliquot of the gas was withdrawn and analysed on a 200 ft $\times$ 0.01 inch i.d. stainless-steel hexadecane-hexadecene Kel F capillary (WCOT) column². This column will separate most alkanes and alkenes in the C_4 – C_7 hydrocarbon range as well as compounds with functional groups such as thio-ethers. Compounds were identified by retention time, by co-injection of standards and by gas chromatography-mass spectrometry. All of the compounds found in these experiments had previously been found in Recent sediments^{1–4}. Limits of detection by this method are 0.003 μg hydrocarbon per litre of medium.

The hydrocarbons formed in these experiments are listed in Table 1. None of these hydrocarbons was recovered in the blank runs including those with bacteria but no substrate and those

Table 1 Yield of C₄–C₇ hydrocarbons from microbial degradation of terpenoids

Mixed culture	Substrate*	Products	Yield† (µg per litre medium)
Metal cans			
Anaerobes	Farnesol	Isopentane	3
		3-Methylpentane	1.5 ± 0.3‡
Anaerobes	β-Carotene	2-Methylpentane	19
		3-Methylpentane	16 ± 2.5‡
		Toluene	35
Serum bottles			
Anaerobes	Farnesol	1-Butene	12
		3-Methylpentene	11
Anaerobes	β-Carotene	Methylbutene	14
Anaerobes	α-2-Pinene	Methylbutene	15
Aerobes (7 days)	Farnesol	1-Butene	141
Anaerobes (8 days)		2-Hexene	9
Aerobes (7 days)	β-Carotene	1-Hexene	30
Anaerobes (8 days)			
Aerobes (7 days)	α-2-Pinene	Methylpentene	17
Anaerobes (8 days)		2,2-Dimethylbutane	21

* About 0.5–1.5 mg of substrate per ml of medium.

† Detection limit 0.003 µg per litre medium.

‡ Standard deviation from duplicate cultures.

with substrate but no bacteria. Small amounts of methane, ethane, propane and butanes were found in some of the samples, but these were also in blanks that did not contain the terpenoids, so their presence was not considered a result of terpene degradation. Also, the samples run in the serum bottles with anaerobic bacteria, but no terpenoids, yielded 50 µg per litre of dimethylsulphide and 25 µg per litre of CS₂ (detected by gas chromatography). When farnesol was added, these yields increased up to 60 µg and 200 µg per litre, respectively.

The most significant conclusion is that the anaerobic degradation process formed only a few specific hydrocarbons from each terpene. Only alkanes and toluene were formed in the metal cans, whereas alkenes were the main products in the glass serum bottles. The kinetics of alkene formation by the anaerobes is possibly faster than that of alkane formation, but is inhibited by the metal in the can. The formation of branched pentanes from either farnesol or β-carotene is conceivable through the breaking of a carbon–carbon bond in the beta position to the double bonds. It is more difficult to understand the formation of the toluene although methylcyclohexene might be a reasonable precursor⁴. β-Carotene will aromatize to form toluene when heated for 2 weeks at 119 °C (ref. 8). However, when heated at 90 °C for 15 min in the conditions of our experiments, no toluene was found within our detection limit (0.003 µg per litre medium). The high yield of toluene in Table 1 only resulted when anaerobes were present in the can. The reason for formation of toluene in the cans but not in the bottles is unclear, but may be due to some effect of the metal in the can. Controls showing no toluene production were: (1) blanks having all substrates including carotene but no bacteria; and (2) bacteria with all carbon sources except carotene. Thus, toluene production due to a metal-catalysed chemical reaction has been ruled out. We did not do aerobic experiments with the metal cans so we cannot conclude that toluene forms exclusively in anaerobic environments.

The anaerobic culturing in the serum bottles yielded mainly branched alkenes. The experiments with aerobes followed by anaerobes yielded mainly straight-chain alkenes and one gem-dimethyl alkane, 2,2-dimethylbutane. The formation of this compound from α-2-pinene could result from breaking off the 4-carbon ring with the quaternary carbon atom in α-pinene.

The results of these preliminary microbial degradation experiments fit surprisingly well with our studies of hydrocarbon distributions in marine sediments. In the strongly reducing environment of Walvis Bay, we found that toluene constituted

70% of the C₄–C₇ hydrocarbons in the surface sediments⁴. The present laboratory experiments support the concept that toluene can be an anaerobic degradation product, and show that the gem-dimethyl alkanes, such as neopentane and 2,2-dimethylbutane dominate in sediments where the overlying waters (and topmost sediments) are oxidizing with deeper sediments reducing (Arabian Sea)³. These same environments also produced a diverse assemblage of alkenes, including straight-chain pentenes and hexenes. Such compounds seemed to arise from a combination of both aerobic and anaerobic microbial degradation of organic substrates, possibly together with low-temperature chemical reactions. Whelan *et al.*⁴ have discussed in detail possible chemical reactions leading to the C₄–C₇ hydrocarbons, such as alcohol dehydration and allylic free radical cleavage. It has also been pointed out that bacterial reworking of algae forms an amorphous biomass high in bacterial bodies that is more readily converted to hydrocarbons in the C₁₅₊ range⁹. However, we found no references to C₄–C₇ hydrocarbon formation by bacterial reworking.

We are not arguing that these products represent major metabolic paths in the organism. The trace amounts of substrate transformed suggest that these paths probably represent reactions which are incidental to the bacteria. However, the small (p.p.m.–p.p.b.) levels of compounds found in these experiments and in our Recent sediment analyses are comparable so that these microbial processes may explain one source of the compounds in Recent sediments. This is a valuable finding because the presence of a particular set of these molecules in more deeply buried cold sediments may provide information about the nature of the sediment–water interface (oxic or anoxic) when the sediment was buried in past geological time.

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- Hunt, J. M. *Nature* **245**, 411 (1975).
- Whelan, J. K. *Init. Rep. DSDP 47a*, 531 (1979).
- Hunt, J. M. & Whelan, J. K. *Org. Geochem.* **1**, 219 (1979).
- Whelan, J. K., Hunt, J. M. & Berman, J. *Geochim. cosmochim. Acta* (in the press).
- Ferry, J. G. & Wolfe, R. S. *Microbiology* **107**, 33 (1976).
- Lyman, J. & Fleming, R. H. *J. Mar. Res.* **3**, 134 (1940).
- Willingham, C. A. & Oppenheimer, C. H. *J. Bact.* **88**, 541 (1964).
- Day, W. C. & Erdman, J. G. *Science* **141**, 808 (1963).
- Lijmbach, G. M. G. *Proc. 9th World Petroleum Congr.* **2**, 357–369 (1975).

The principal eyes of a jumping spider have a telephoto component

David S. Williams & Peter McIntyre

Department of Neurobiology, Research School of Biological Sciences, Australian National University, Canberra, Australia

Jumping spiders are a cosmopolitan family (Salticidae) of predators that can make visual discrimination between prey and mates^{1,2}. This task is mediated through the anterior median eyes, described by Land³ as consisting of a corneal lens and a motile retina that comprises four layers of receptors embedded in a matrix. The retinal matrix contains a pit distal to the receptors and symmetrically centred on-axis. We have now found that in *Portia fimbriata* (Doleschall) and some other species, this pit has a refracting interface that increases the focal length of the eye beyond its axial length, thereby magnifying the retinal image and increasing visual resolving power above that possible with only a corneal lens. The most effective part of the conical pit is its rounded apex, which augments the corneal lens to provide a telephoto system that increases the overall focal length by about 1½ times. This mechanism is of particular value to small spiders like *P. fimbriata*, for the possible axial length of their eyes is constrained by the small size of their prosomae (Fig. 1).

The inside of the pit of the anterior median eye of *P. fimbriata* is structureless and must have a refractive index (n) close to that of saline, about 1.336 (Fig. 2). In glutaraldehyde-fixed material, cut while frozen, the matrix behind the pit was found by interferometry to have a uniform n , a value of ~ 1.40 being obtained from unfixed material. The rounded apex of the pit is about $20\text{ }\mu\text{m}$ in diameter; the most central region is approximately spherical, with radius of curvature $\sim 5\text{ }\mu\text{m}$, and thus acts as the diverging lens of the telephoto system (Fig. 3), with power $(1.40-1.336)/(-5\times 10^{-6}) = -12,800$ dioptres. In longitudinal profile, the rounded region is less curved beyond about $4\text{ }\mu\text{m}$ from the axis and merges into straight sides (Fig. 2). Such an aspherical surface may well give a better image over a wider aperture than a spherical one. The curvature of the pit varies greatly among the different species of salticids and in some cases is so slight that the pit will have negligible power.

From a corneal lens (diameter $810\text{ }\mu\text{m}$) of *P. fimbriata*, dissected out and hung from a drop of saline ($n = 1.336$) to simulate *in situ* conditions^{4,5}, a front focal length of $-1,273\text{ }\mu\text{m}$, that is, a power of $+786$ dioptres, was measured (in green light, as was used for the measurements of n). The second principal plane of the corneal lens was found nearly to coincide with the front surface of the lens, indicating that the air/cornea interface provides most of the refracting power. The remaining parameter necessary for determination of the optical properties of the telephoto system is the distance, d , between the corneal lens (more precisely, its second principal plane) and the pit. d was deduced from ophthalmoscopy, using an apparatus similar to that devised by Land^{3,6}. By finding the position in space of the image of a recognizable part of the retina (in this case the virtual image of parts of the receptor cells in the characteristically organized fourth receptor layer, which on-axis lies $25\text{ }\mu\text{m}$ behind the pit), d can be calculated from standard thick-lens formulae⁷. (Ideally the properties of the complete dioptric system could be determined directly from the magnification of the ophthalmoscopic image, but this was prevented by uncertainty about exactly which parts of the receptor cells in layer 4 provide the image.) The value determined for d ($=1,664\text{ }\mu\text{m}$) is to be compared with the focal length in image space of the corneal lens ($1,273\times 1.336 = 1,701\text{ }\mu\text{m}$). To achieve a reasonable magnification without unduly increasing image distance, the pit must be a short distance inside the focal point of the corneal lens. This condition seems to be satisfied.

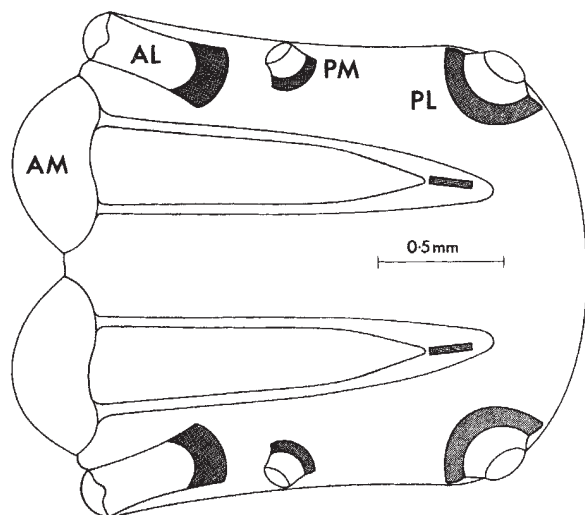


Fig. 1 Diagrammatic horizontal section of the dorsal anterior part of the prosoma of *P. fimbriata*, showing the positions of the anterior (A) and posterior (P) median (M) and lateral (L) eyes. This eye-bearing portion of the prosoma projects dorsally. In the dorso-ventral plane, the posterior eyes lie just above the anterior median retinae.

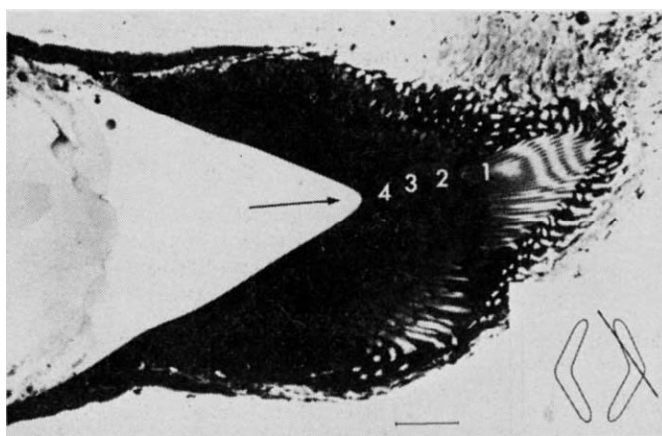


Fig. 2 Longitudinal section of an anterior median retina in *P. fimbriata* including part of one arm of its 'boomerang' structure. The material was fixed in glutaraldehyde and OsO_4 , embedded in Araldite and stained with toluidine blue. Scale bar, $50\text{ }\mu\text{m}$. The interior of the pit (arrow) is structureless (the glass cells visible on the left side of the figure do not extend into the pit), but the surrounding retinal matrix has stained densely and homogeneously. Receptor layers are numbered. Horizontally, across the narrow centre of the retina, the distal ends of the layer 1 receptors are arranged as a staircase, such that on the lateral side of the retina they are $25\text{ }\mu\text{m}$ more distal than on the medial. The overall radius of curvature of the retina in a dorso-ventral plane is far smaller than would be expected if the receptors were organized around the corneal lens. This indicates further that the retina is organized according to the combined properties of the corneal lens and the pit. Inset: Diagram outlining the two anterior median retinae in transverse section at the level of layer 1. The line indicates the plane of the longitudinal section.

Using the above parameters, the position of focus, F' , of the telephoto system for an object at infinity is $59\text{ }\mu\text{m}$ behind the pit, corresponding to the distal on-axis region of the second receptor layer, and the magnification achieved by the pit lens is 1.54 (see Fig. 3). The following supports this finding. Comparative ultrastructural studies of salticid principal retinae⁸ show that (1) layer 1 receptors in the central region form a high-resolution mosaic whose rhabdom organization implies that the individual rhabdoms are light guides; (2) layer 2 receptors form a coarse central mosaic with probable optical coupling between receptors. In *Plexippus*, recordings from dye-injected cells have identified both layers 1 and 2 as green receptors with peak responses at $\sim 520\text{ nm}$ (ref. 9). Cells peaking in the green have also been found previously¹⁰; discrepancies between these results and other published data¹¹ will be discussed elsewhere⁹. Some salticids, including *P. fimbriata*, seem to discriminate between conspecifics and prey at distances of $15\text{--}20\text{ cm}$ (refs 12, 13); *Trite planiceps* Simon, a salticid that we find has a significant pit, but lower overall image magnification than *P. fimbriata*, is known to make discriminations at distances of 20 cm (ref. 12). Thus, a 'best solution' for the dioptric system must provide focused images of objects, $\sim 20\text{ cm}$ in front of the spider, at the tips of the rhabdoms of layer 1, when $\lambda = 520\text{ nm}$. For F' near the distal ends of the layer 2 receptors, the distal ends of those of layer 1 (Fig. 2) receive best images from objects $15\text{--}29\text{ cm}$ in front of the spider and have a total depth of field of 0 to ∞ . The depth of field is estimated by compounding the horizontal 'staircase' tiering of the distal ends of layer 1 receptors (which gives the $15\text{--}29\text{-cm}$ range) with the depth of focus due to diffraction at the aperture of the eye⁷ (see below).

The retina of an anterior median eye is boomerang-shaped (Fig. 2), measuring only $250\text{ }\mu\text{m}$ dorso-ventrally and $25\text{ }\mu\text{m}$ wide—a size reduction necessary to accommodate the eye's long axis³ (Fig. 1). At the level of layer 1, the receptors in the centre of

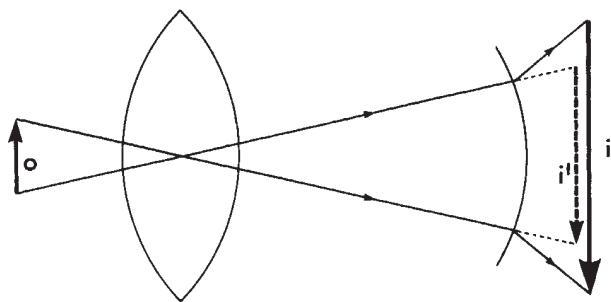


Fig. 3 General schematic illustration of the enlargement of an image by the diverging back element of a telephoto system. *o*, object; *i*, image; *i'*, image in absence of diverging surface. The magnification, *m*, effected by the pit lens in *P. fimbriata* is given by⁷

$$m = \frac{\text{size of } i}{\text{size of } i'} = P_L/P_T = \left[1 + \frac{P_P(1 - \bar{d}P_L)}{P_L} \right]^{-1}$$

where P_T is the power of the telephoto lens system, and P_L , P_P the powers of its components, the converging corneal lens and the diverging pit lens respectively. $\bar{d} = d/1.336$, with d as defined in the text. This magnification requires an increase in the axial length of the eye of only 22 μm .

the retina have oval transverse profiles (minimum size $0.8 \mu\text{m} \times 1.5 \mu\text{m}$) and form an hexagonal array with a minimum receptor-row spacing of $1.4 \mu\text{m}$ (ref. 8), corresponding to a visual angle of 2.4 arc min. (This corresponds to a distance of 0.12 mm at 20 cm in front of the spider.) The resolving ability of a receptor mosaic depends on the angular subtense between neighbouring receptors, that is, on the receptor spacing and focal length of the eye. The receptor spacing at the centre of layer 1 is near the limit imposed by the wave nature of light, because optical crosstalk¹⁴ between the 80–100- μm long rhabdoms would probably be excessive were they much closer together. Therefore, the only way to enhance the anatomical resolving power of the eye is to increase its focal length. Image magnification by the pit achieves this, albeit at the cost of narrowing the field of view of the central retina. About 100 receptors in the centre of layer 1 lie behind the curved region of the pit in a circle of diameter 21 μm —most of the width of the central part of layer 1. In total they have a field of view of 0.6° .

Vertically, the field of view of the eye is extended by the dorsal and ventral extremities of the retina. Light reaching these regions must pass through the sides of the pit which, in longitudinal section, are straight beyond 10 μm from the optic axis and inclined at 30° to it. They produce a range of magnifications of 1.1–1.25, depending on the angle at which rays are incident. Magnification by the whole pit is therefore greatest on-axis, owing to the lens component, and decreases to a negligible amount for very off-axis light. Because off-axis magnification is different for different angles of incidence on the sides of the pit, off-axis focus will be blurred. This poorer image quality is reflected by the receptor mosaic off-axis in layers 1 and 2 where the rhabdoms are larger ($\sim 10 \mu\text{m}$ in diameter) and practically contiguous.

A jumping spider's field of view is extended horizontally by its lateral eyes which, in conjunction with the vertical extremities of the anterior median retinae, allow peripheral detection. Peripheral detection of an object initiates body and anterior median retinal movements that bring images of the object to lie in the centres of the retinae and maintain them there during fixation⁶.

Land³ suggested that the resolution of the anterior median eyes of two salticid species is probably limited by spherical aberration of their corneal lenses. Our re-examination of the lenses of *P. fimbriata* and several other salticids in a hanging drop and by interference microscopy shows that this defect is corrected by a graded refractive-index distribution in the lens, as in some nocturnal spiders^{5,15,16}. Probably, then, the anterior median corneal lenses in salticids are diffraction limited. If this is true of the telephoto system of *P. fimbriata* as a whole, the

maximum or cut-off spatial frequency passed by it is 760 rad^{-1} (refs 17–19). (The limiting aperture is a ring of pigment, 350 μm in diameter and 315 μm behind the corneal surface. The position of focus is taken as the distal ends of the receptors in layer 1. $\lambda = 520 \text{ nm}$.) The highest spatial frequency that can be reconstructed by layer 1 with minimum receptor-row spacing 1.4 μm and distance 1,980 μm from the posterior nodal point of the telephoto system is $1,980/2.8 = 710 \text{ rad}^{-1}$. Therefore, the receptor spacing in the centre of layer 1 seems to be well matched to the telephoto system. In the absence of the pit, the highest frequency reconstructable by the retina would be only 420 rad^{-1} , which is well below the cut-off frequency for the lens of 850 rad^{-1} .

A parallel to the pit of *P. fimbriata* is the deep convexiculate fovea found in some vertebrates, notably falconiform birds²⁰. It has been proposed that this fovea magnifies the retinal image^{20,21} and acts as a focus indicator²². The latter is an implausible function for the pit in *P. fimbriata*, for as in other salticids³, ophthalmoscopy shows that the eye does not accommodate. Aerodynamic constraints require the heads of birds to be small. Both a group of vertebrates and an invertebrate have therefore adopted the same strategy to improve visual acuity despite a restricted cephalic space.

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- Crane, J. *Zoologica* **34**, 159–214 (1949).
- Drees, O. *Z. Tierpsychol.* **9**, 169–209 (1952).
- Land, M. F. *J. exp. Biol.* **51**, 443–470 (1969).
- Homann, H. *Z. vergl. Physiol.* **7**, 201–268 (1928).
- Blest, A. D. & Land, M. F. *Proc. R. Soc. B* **196**, 197–222 (1977).
- Land, M. F. *J. exp. Biol.* **51**, 471–493 (1969).
- Longhurst, R. S. *Geometrical and Physical Optics* (Longman, London, 1973).
- Blest, A. D., McIntyre, P. & Williams, D. S. (in preparation).
- Blest, A. D., Hardie, R. C., McIntyre, P. & Williams, D. S. (in preparation).
- De Voe, R. D. *J. gen. Physiol.* **66**, 193–207 (1975).
- Yamashita, S. & Tateda, H. *J. comp. Physiol.* **105**, 29–41 (1976).
- Forster, L. M. *N.Z. J. Zool.* **6**, 79–93 (1979).
- Jackson, R. R. & Blest, A. D. (in preparation).
- Snyder, A. W. *J. opt. Soc. Am.* **62**, 1267–1277 (1972).
- Williams, D. S. *Z. Naturforsch.* **34c**, 463–469 (1979).
- Blest, A. D., Williams, D. S. & Kao, L. *Cell Tissue Res.* **211**, 391–403 (1980).
- Hopkins, H. H. *Proc. R. Soc. A* **231**, 91–103 (1955).
- Born, M. & Wolf, E. *Principles of Optics*, 5th edn (Pergamon, Oxford, 1975).
- Snyder, A. W. *J. comp. Physiol.* **116**, 161–182 (1977).
- Walls, G. L. *The Vertebrate Eye* (Cranbrook Institute of Science, Bloomfield Hills, Michigan, 1942).
- Snyder, A. W. & Miller, W. H. *Nature* **275**, 127–129 (1978).
- Harkness, L. & Bennet-Clark, H. C. *Nature* **272**, 814–816 (1978).
- Williams, D. S. & McIntyre, P. *Proc. Aust. physiol. pharmac. Soc.* 182P (1980).

Correlative genetic variation in natural populations of cats, mice and men

Stephen J. O'Brien,* Mitchell H. Gail†
& David L. Levin†

* Laboratory of Viral Carcinogenesis, National Cancer Institute, National Institute of Health—Frederick Cancer Research Center, Frederick, Maryland 21701

† Biometry Branch, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20205

The study of the extent and basis of gene-enzyme variation has long been a principal concern of population genetics. Numerous surveys have indicated considerable amounts of genetic variation detectable in natural populations, with few exceptions^{1–14}. The variances of average heterozygosities (*H*) between species and among populations within species are large, prompting Lewontin to emphasize the importance of large gene sample sizes⁷ and Selander to encourage analysis of variation of

homologous gene-enzyme systems when making species comparisons⁸. We present here a comparative genetic analysis of electrophoretic variation at 57 homologous biochemical loci of cats, mice and men. The distribution of polymorphism among the sampled loci in the three species was nonrandom. A large group of sampled loci (60%) were monomorphic in all three species, whereas a second group (30%) of the loci were polymorphic in two or more species. This conservation of the tolerance of genetic polymorphism is apparently more a characteristic of a particular locus than of the vertebrate species or of the genome. The current hypotheses for classifying polymorphic and monomorphic loci in terms of physiological and physical enzyme characteristics have been re-examined.

Table 1 shows a list of per locus heterozygosities ($h = 1 - \sum x_i^2$ where x_i is the frequency of the i th allele at a locus) of 57 gene-enzyme systems of one feline, two murine and two human populations. The loci encode homologous enzymes of the species, based on enzymatic, histological and molecular enzyme characteristics^{6,15-17} as defined elsewhere¹⁵. The nomenclature of the human and feline loci conforms to the recommendations given in ref. 18; similarly the mouse nomenclature conforms to recommendations of ref. 19. The 57 loci are those which have been studied in either the cat population⁶ or the two mouse populations¹⁰, and also in human populations^{2,20} and for which enzymatic homology has been tentatively established. The feline population is a feral farm cat population studied at 55 loci⁶. The human data are from Harris²⁰ in almost all cases and represent cumulative estimates summarized by Harris and Hopkinson^{2,15}. These estimates of human loci agree well with those presented by Nei and Roychoudhury²¹. In a few cases (*GALA* and *ALB*) the latter estimates are used because these loci are not included by Harris and Hopkinson^{2,15,20}.

The LC and BC mouse populations are feral mouse populations collected in Southern California in 1977 and have been described in detail elsewhere^{10,22}. To demonstrate that these are representative mouse populations, they were compared with previous surveys, that is, those of the island east of Jutland, Denmark population⁵; the Hallowell Farm, California population⁴; the British mainland population²³, and the outbred Swiss mouse colony at the Eppléy Institute, Omaha, Nebraska²⁴. The 12 polymorphic mouse loci in LC and/or BC listed in Table 1 were all polymorphic in previous mouse surveys. Four of the murine loci *Es-10*, *Glo-1*, *Mod-2*, and *Gpd-1* that are monomorphic in LC and BC, but fall in the polymorphic cluster (1-22 in Table 1), are also polymorphic in other mouse surveys^{4,5,23-25}.

The 57 loci in Table 1 are listed according to the extent of polymorphism seen in the three species. The first 22 loci are polymorphic in at least 1 of the 3 species, and, of these, 14 (25% of the total) are polymorphic in 2 species. The estimate rises to 30% if *Glo-1*, *Mod-2*, *Es-10* and *Gpd-1* (which are monomorphic in LC and BC mice but vary between inbred strains and in Swiss, Hallowell Farm and Jutland Island mice) are included. If the distribution of allozyme polymorphism in two species were independent, the frequency of systems which are polymorphic in any two species would be equal to the product of their individual polymorphism frequencies. For example, in our cat sample⁶ (Table 1), the proportion of loci tested which were polymorphic (P_{cat}) was 0.22 (12/55 loci). The value for the California mice (P_{mouse}) sampled was 0.20. Thus, the expected frequency of loci which are polymorphic in both species, if the estimates were independent, equals $P_{cat} \times P_{mouse} = 0.04$. The observed frequency of systems which vary in both cat and mouse was 0.14 (5 of 35 loci), nearly 4 times greater than expected.

Table 1 Comparison of biochemical allelic variation of homologous loci of cat, mouse and man

Human/feline gene	Homologous mouse gene	Subunit number	Subunit molecular weight ($\times 10^{-3}$)	Enzyme class	h = heterozygosity				
					<i>Felis catus</i> (Mid Atlantic US)	<i>Homo sapiens</i>		<i>M. musculus domesticus</i>	
						(Caucasian)	(Negro)	(LC)	(BC)
1. <i>PGD</i>	<i>Pgd</i>	2	52	N	0.47	0.04	0.09	0.24	0.30
2. <i>HBB</i>	<i>Hbb</i>	4*	16	—	0.30	0.0	0.15	0.32	0.44
3. <i>ESD/ES5</i>	<i>Es-10</i>	2	28	V	0.34	0.18	0.18	0.0	0.0
4. <i>PGM3</i>	—	1	53	R	0.38	0.38	0.45	—	—
5. <i>FUCA</i>	—	1	50	—	0.20	0.38	0.13	—	—
6. <i>GDH2</i>	<i>Gpd-1</i>	2	—	—	0.49	—	—	—	—
7. <i>PEPA</i>	<i>Pep-1</i>	2	46	V	0.48	0.38	0.31	0.0	0.0
8. <i>ADA</i>	<i>Ada</i>	1	34	R	0.53	0.10	0.04	0.0	0.0
9. <i>—/ES1</i>	<i>Es-2</i>	1	55	V	0.35	—	—	0.32	0.50
10. <i>GPI</i>	<i>Gpi-1</i>	2	62	R	0.10	0.0	0.0	0.0	0.05
11. <i>ME1</i>	<i>Mod-1</i>	4	60	R	0.11	0.0	0.0	0.30	0.42
12. <i>GSR</i>	<i>Gr-1</i>	2	50	—	0.0	0.50	0.48	0.50	0.50
13. <i>GPT</i>	<i>Gpt-1</i>	2	50	—	0.0	0.50	0.26	0.40	0.06
14. <i>PGM1</i>	<i>Pgm-2</i>	1	51	R	0.0	0.38	0.32	0.0	0.48
15. <i>GOT 2</i>	<i>Got-2</i>	2	46	N	0.0	0.04	0.15	0.47	0.42
16. <i>ME 2</i>	<i>Mod-2</i>	4	60	R	0.0	0.18	0.0	0.0	0.0
17. <i>GLO1</i>	<i>Glo-1</i>	—	—	—	0.0	0.49	0.40	0.0	0.0
18. <i>PFK</i>	—	4	100	R	0.25	0.0	0.0	—	—
19. <i>G6PD</i>	<i>G6PD</i>	2	53	R	0.0	0.0	0.46	0.0	0.0
20. <i>ACP1</i>	<i>Acp-1</i>	1	15	V	0.0	0.48	0.45	0.0	0.0
21. <i>GOT1</i>	<i>Got-1</i>	2*	46	N	0.0	0.0	0.0	0.38	0.0
22. <i>ID1</i>	<i>Id-1</i>	2	48	N	0.0	0.0	0.0	0.51	0.0
23. <i>APRT</i>	<i>Aprt</i>	2	16	—	0.0	0.0	0.0	0.0	0.0
24. <i>AK1</i>	<i>Ak-1</i>	1	22	R	0.0	0.04	0.02	0.0	0.0
25. <i>AK2</i>	<i>Ak-2</i>	1	40	R	—	0.0	0.0	0.0	0.0
26. <i>ALDA</i>	—	4*	39	N	0.0	0.0	0.0	—	—
27. <i>ADH1</i>	<i>Adh-2</i>	2*	40	R	—	0.0	0.0	0.0	0.0
28. <i>CPKB</i>	—	2*	41	—	0.0	0.0	0.0	—	—
29. <i>DIA1</i>	—	1	30	—	0.0	0.0	0.0	—	—
30. <i>ESA/ES7</i>	<i>Es-s</i>	—	—	V	0.0	0.0	0.0	0.0	0.0
31. <i>GALA</i>	—	2	—	—	0.0	0.0	0.0	—	—
32. <i>GALB</i>	<i>Bgs</i>	1	72	—	0.0	0.0	0.0	—	—
33. <i>GDH1</i>	—	2	111	—	0.0	0.0	—	—	—
34. <i>GAPD</i>	<i>Gapdh</i>	4	36	R	—	—	0.0	0.0	0.0
35. <i>GUSB</i>	<i>Gus</i>	—	—	—	0.0	0.0	0.0	0.0	0.0

(Table 1 continued overleaf)

Table 1 continued

Human/feline gene	Homologous mouse gene	Subunit number	Subunit molecular weight ($\times 10^{-3}$)	Enzyme class	h = heterozygosity				
					<i>Felis catus</i> (Mid Atlantic US)	<i>Homo sapiens</i> (Caucasian)	<i>Homo sapiens</i> (Negro)	<i>M. musculus domesticus</i> (LC)	<i>M. musculus domesticus</i> (BC)
36. <i>HEXA</i>	—	—	—	—	0.0	0.0	—	—	—
37. <i>HEXB</i>	—	—	—	—	0.0	0.0	—	—	—
38. <i>HK1</i>	<i>Hk-1</i>	1	107	R	0.0	0.0	0.0	0.0	0.0
39. <i>HPRT</i>	<i>Hprt</i>	3	26	—	—	0.0	0.0	0.0	0.0
40. <i>IDH2</i>	<i>Idh-2</i>	—	—	R	0.0	0.0	0.0	0.0	0.0
41. <i>LHDA</i>	<i>Ldh-1</i>	4*	35	N	0.0	0.0	0.0	0.0	0.0
42. <i>LDHB</i>	<i>Ldh-2</i>	4*	35	N	0.0	0.0	0.0	0.0	0.0
43. <i>MDH1</i>	<i>Mor-2</i>	2*	35	N	0.0	0.0	0.0	0.0	0.0
44. <i>MDH2</i>	<i>Mor-1</i>	2	35	N	0.0.0	0.0	0.0	0.0	0.0
45. <i>MPI</i>	<i>Mpi-1</i>	1	43	—	0.0	0.02	—	0.0	0.0
46. <i>NP</i>	<i>Np-1</i>	3	28	—	0.0	0.0	0.0	0.0	0.0
47. <i>PEPB</i>	<i>Pep-2</i>	1	55	V	0.0	0.0	0.0	0.0	0.0
48. <i>PEPC</i>	<i>Dip-1</i>	1	64	V	0.0	0.02	0.01	0.0	0.0
49. <i>PEPD</i>	<i>Pep-4</i>	2	50	V	0.0	0.0	0.04	0.0	0.0
50. <i>PEPS</i>	<i>Pep-7</i>	—	—	V	—	0.0	0.0	0.0	0.0
51. <i>PGM2</i>	<i>Pgm-1</i>	1	61	R	—	0.0	0.02	0.0	0.0
52. <i>PP</i>	<i>Pyp</i>	2	23	—	0.0	0.0	0.0	0.0	0.0
53. <i>SOD1</i>	<i>Sod-1</i>	2*	16	V	—	0.0	0.0	0.0	0.0
54. <i>SOD2</i>	<i>Sod-2</i>	4	18	V	0.0	0.0	0.0	0.0	0.0
55. <i>SORD</i>	—	4	38	N	0.0	0.0	0.0	—	—
56. <i>TPI</i>	—	2	26	N	0.0	0.0	0.0	—	—
57. <i>ALB</i>	<i>Alb-1</i>	—	12	—	0.0	0.0	0.0	—	—

Enzyme homology (first 2 columns) is based on the following characteristics: (1) substrate specificity; (2) tissue distribution; (3) subcellular organelle association; (4) formation of heteropolymer in somatic cell hybrids; (5) cofactor affinity, and (6) relative electrophoretic mobility. The specific characteristics of each system are described elsewhere^{6,15-17}. Subunit number and molecular weight estimates were obtained from refs 39 and 40. Enzyme class after the definitions of Johnson³². V, variable substrate class; R, regulatory enzyme class; N, non-regulatory enzyme class. Heterozygosity (h) at each locus (assuming Hardy-Weinberg equilibrium) $= 1 - \sum x_i^2$ where x_i is the frequency of the i th allele in the population at the locus. A locus is considered to be polymorphic if $h \geq 0.05$. If two h measurements are available, as for LC and BC mice, the species is said to be polymorphic at that locus if either h value equals or exceeds 0.05. The data are derived from published surveys of feline⁶, human^{2,20}, and murine¹⁰ populations. To test for nonrandom distribution of polymorphic loci in the three species sampled, consider only those ($T = 34$) loci for which complete data are available. If P_c , P_h , and P_m are the numbers of polymorphic loci in the cat, human and murine samples, and M_c , M_h , and M_m are the corresponding numbers of monomorphic loci, then, under independence, the expected number of loci polymorphic in all species (PPP) is $(P_c/T)(P_h/T)(P_m/T) T = 0.7872$ and the expected number of loci monomorphic in all three species (MMM) is $(M_c/T)(M_h/T)(M_m/T) T = 11.7716$. The expected number of concordant loci ($PPP + MMM$) is 12.5588. The observed value is $2 + 17 = 19$ concordant loci. The values $P_c = 7$, $P_h = 13$, $P_m = 10$, $M_c = 27$, $M_h = 21$ and $M_m = 24$ were obtained from Table 1 using the definition of monomorphic $h < 0.05$ given above. To determine whether the observed number of concordant PPP matches (Y_1) and concordant MMM matches (Y_2) were significantly greater than expectation, the joint distribution of Y_1 and Y_2 was computed. The number X of PP matches for cat and mouse has the hypergeometric mass function $P(X) = C(P_c, X)C(M_c, P_m - X)/C(T, P_m)$ where $T = P_c + M_c$ and $C(n_1, n_2) = n_1!/(n_1!(n_2 - n_1)!)$. Possible values of X are determined from the 2×2 table with margins P_c , M_c and P_m , M_m . Given X , the conditional joint distribution of Y_1 and Y_2 is given by $P(Y_1, Y_2|X) = C(X, Y_1)C(b, Y_2|C(d, P_h - Y_1 + Y_2 - b))/C(T, P_h)$ where $b = T + X - P_c - P_m$ and $d = P_c + P_m - 2X$. Possible values of Y_1 and Y_2 are determined, given X , from the 2×3 table with margins X , b , d and P_h , M_h . The joint probability mass function $P(Y_1, Y_2)$ is obtained for each Y_1 and Y_2 as the sum over all possible X of terms $P(X)P(Y_1, Y_2|X)$. From this distribution it follows that $P(Y_1 \geq 2) = 0.17410$, $P(Y_2 \geq 17) = 0.00106$ and $P(Y_1 + Y_2 \geq 19) = 0.00396$. Thus the number (Y_1) of concordant PPP is not significantly in excess of expectation, but the numbers of MMM (Y_2) and total ($Y_1 + Y_2$) concordances are significantly greater than predicted by chance. To check these calculations, exact means, variances, and covariances of Y_1 and Y_2 were computed using indicator functions and found to agree with those moments of the distribution $P(Y_1, Y_2)$. These moment calculations extend the two-dimensional analysis of Everitt⁴¹ to three or more dimensions. In addition, simulation studies confirmed the calculations of $P(Y_1 \geq 2)$, $P(Y_2 \geq 17)$ and $P(Y_1 + Y_2 \geq 19)$ above. If the definition of monomorphic is changed from the widely used value $h < 0.05$ to the very stringent value $h < 0.01$, the number of MMM concordances is reduced to $Y_2 = 13$ whereas Y_1 is unchanged. Using the corresponding parameters $P_c = 7$, $M_c = 27$, $P_h = 17$, $M_h = 17$, $P_m = 10$, $M_m = 24$, we find $P(Y_1 \geq 2) = 0.2716$, $P(Y_2 \geq 13) = 0.02627$ and $P(Y_1 + Y_2 \geq 15) = 0.03586$. Thus, even with this unusually stringent definition of monomorphic, the excess in Y_2 and $Y_1 + Y_2$ is statistically significant.

* Indicates those enzyme systems which are inter-locus heteropolymers.

A similar comparison of cat and human^{2,6,20} gave an expected overlap frequency of $P_{cat} \times P_{human} = 0.22 \times 0.28 = 0.06$; the observed frequency (Table 1) was $7/46 = 0.15$. The mouse-human expectation was 0.06, and the observed match frequency 0.15. The expected frequency of systems polymorphic in all three species assuming random distribution was $P_c \times P_h \times P_m = 0.01$. The actual frequency of triple matches was 2 in 36 (0.06). The frequency of polymorphic triplets may actually be higher than indicated because four mouse loci, whose homologues are polymorphic in cat and man, are likely to be polymorphic in mouse, but were not considered as such in our analysis. *Es-10* and *Gpd-1* vary between inbred strains, presumably reflecting allelic variation in their founders²⁵. Human and feline *PGM3* have no murine counterpart, although *PGM3* is polymorphic in man, cats and dogs^{2,6,26}. *FUCA* is polymorphic in cat and man, but has not yet been typed in any murine population. A three-dimensional statistical analysis of the distribution of polymorphic against monomorphic loci in Table 1 revealed a

significant ($P = 0.004$) excess of monomorphic triplets (that is, loci monomorphic in each species) and polymorphic triplets (loci polymorphic in all three species) over expectation. The statistical methods are shown in Table 1 legend.

The interspecies correlation of the loci which are polymorphic and those which are monomorphic emphasizes the notion that genic polymorphism is a characteristic of certain homologous loci, but not of others in these different species, which have been isolated in evolutionary time for 80 Myr. Certain loci, then, seem to have an adaptive tolerance to accommodate genetic variation whereas others do not, presumably due to selective constraints which physiologically or functionally restrict significant introduction of gene-enzyme variants into a population. An extension of this situation would be the possibility of selective advantage in polymorphism *per se* at the variant loci themselves, in a situation similar to the major histocompatibility complex (MHC) of all vertebrates thus far studied^{27,28}. The MHC loci are characteristically polymorphic in natural popu-

lations, often with dozens of segregating alleles rendering virtually 100% heterozygosity within the population.

The molecular and physiological basis of the tendency of specific loci to be polymorphic (or not) is not immediately apparent. A number of adaptive and metabolic models have been proposed to be operative in this decision in *Drosophila* and human populations²⁹⁻³⁴. Enzyme characteristics which have been suggested in this determination include: (1) whether an enzyme has broad substrate specificity and uses exogenous environmental substrates compared with enzymes with narrow substrate specificities and which are members of intermediary enzyme pathways²⁹⁻³¹; (2) whether or not an enzyme in a pathway exerts a regulatory role^{32,33}; (3) the structural gene size as estimated by subunit molecular weight^{34,35}; and (4) selective neutrality of the sub-genic portions of polymorphic genes which vary by random mutational substitution³⁶. We have estimated the amount of variation in *h* values which can be explained by the first three selective hypotheses using a one-way analysis of variance (data not shown). Although there are tendencies in each case to conform to the models, in no case can more than 10% of the variation be explained by any one model. Whether selective or non-selective factors contribute inordinately to allozyme variation of these systems is not clear.

Finally, the evolutionary conservation of genetic organization and selective factors has also been observed between these three classes of organisms in comparative genetic mapping^{16,37,38}. Apparently, blocks of genes have been conserved as linkage groups in a number of cases between cats, mouse and man^{16,17,37,38}. The comparative linkage associations and the comparative population structures of these distantly related mammalian genera provide hope for the further understanding of adaptive processes of chromosomal and molecular evolution.

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- Powell, J. in *Evolutionary Biology* Vol. 8 (eds Dobzhansky, T., Hecht, M. K. & Steere, W. C.) 79-119 (Plenum, New York, 1976).
- Harris, H. & Hopkinson, D. A. *Ann. hum. Genet.* **36**, 9-20 (1972).
- Lewontin, R. C. & Hubby, J. L. *Genetics* **54**, 595-609 (1966).
- Selander, R. K. & Yang, S. Y. *Genetics* **63**, 653-667 (1969).
- Selander, R. K., Hunt, W. G. & Yang, S. Y. *Evolution* **23**, 379-390 (1969).
- O'Brien, S. J. *J. Hered.* **71**, 2-8 (1980).
- Lewontin, R. C. *The Genetic Basis of Evolutionary Change* (Columbia University Press, New York, 1974).
- Selander, R. K. in *Molecular Evolution* (ed. Ayala, F.) 21-45 (Sinauer, Massachusetts, 1976).
- Johnson, G. B. in *Molecular Evolution* (ed. Ayala, F.) (Sinauer, Massachusetts, 1976).
- Rice, M. C., Gardner, M. B. & O'Brien, S. J. *Biochem. Genet.* **8**, 915-928 (1980).
- Racine, R. R. & Langley, C. H. *Nature* **283**, 855 (1980).
- Bonnell, M. L. & Selander, R. K. *Science* **184**, 908-910 (1974).
- Cameron, D. G. & Vyse, E. R. *Biochem. Genet.* **16**, 651-657 (1978).
- Manlove, M. N., Baccus, R., Pelton, M. R., Smith, M. H. & Gruber, D. in *Proc. 4th Int. Conf. Bear Research and Management* (ed. Martinka, C. J.) (Wildlife Management Inst., in the press).
- Harris, H. & Hopkinson, D. A. *Handbook of Enzyme Electrophoresis in Human Genetics* (North Holland, Amsterdam, 1976).
- Pearson, P. L. *et al. Cytogenet. Cell Genet.* **25**, 82-95 (1979).
- O'Brien, S. J. & Nash, W. G. (submitted).
- Shows, T. *et al. Cytogenet. Cell Genet.* **25**, 96-116 (1979).
- Committee on Standardization of Genetic Nomenclature for Mice *J. Hered.* **63**, 69-72 (1972).
- Harris, H. *Isozyme Bull.* **10**, 22-25 (1977).
- Nei, M. & Roychoudhury, A. K. *Am. J. hum. Genet.* **26**, 421-443 (1974).
- Gardner, M. B. *Curr. Topics Microbiol. Immun.* **79**, 215-259 (1978).
- Berry, R. J. & Peters, J. *Proc. R. Soc. B* **197**, 485-503 (1977).
- Rice, M. C. & O'Brien, S. J. *Nature* **283**, 157-161 (1980).
- Staats, J. *Cancer Res.* **36**, 4333-4377 (1976).
- Meera Khan, P., Los, W. R. T., van der Does, J. A. & Epstein, R. B. *Transplantation* **15**, 624-628 (1973).
- Klein, J. *Biology of the Mouse Histocompatibility-2 Complex* (Springer, New York, 1975).
- Gotze, D. (ed.) *The Major Histocompatibility System in Man and Animals* (Springer, New York, 1977).
- Gillespie, J. H. & Kojima, K. *Proc. natn. Acad. Sci. U.S.A.* **61**, 582-585 (1968).
- Kojima, K., Gillespie, J. H. & Tobari, Y. N. *Biochem. Genet.* **4**, 627-637 (1970).
- Gillespie, J. H. & Langley, C. H. *Genetics* **76**, 837-848 (1974).
- Johnson, G. B. *Science* **184**, 28-37 (1974).
- Zouros, E. *Nature* **254**, 446-448 (1975).
- Koehn, R. K. & Eanes, W. F. *Evolut. Biol.* **11**, 39-100 (1978).
- Harris, H., Hopkinson, D. A. & Edwards, Y. H. *Proc. natn. Acad. Sci. U.S.A.* **74**, 698-701 (1977).
- Kimura, M. & Ohta, T. *Nature* **229**, 467-499 (1971).
- Lalley, P. A., Minna, J. D. & Franke, U. *Nature* **274**, 160-163 (1978).
- Lalley, P. A., Francke, U. & Minna, J. D. *Proc. natn. Acad. Sci. U.S.A.* **75**, 2382-2386 (1978).
- Hopkinson, D. A., Edwards, Y. H. & Harris, H. *Ann. hum. Genet.* **39**, 383-411 (1976).
- Sober, H. A. *Handbook of Biochemistry, Selected Data for Molecular Biology* (CRC, Cleveland, 1970).
- Everitt, B. S. *Br. J. math. stat. Psychol.* **21**, 97-103 (1968).

Delayed formation of proteoglycan aggregate structures in human articular cartilage disease states

T. R. Oegema Jr

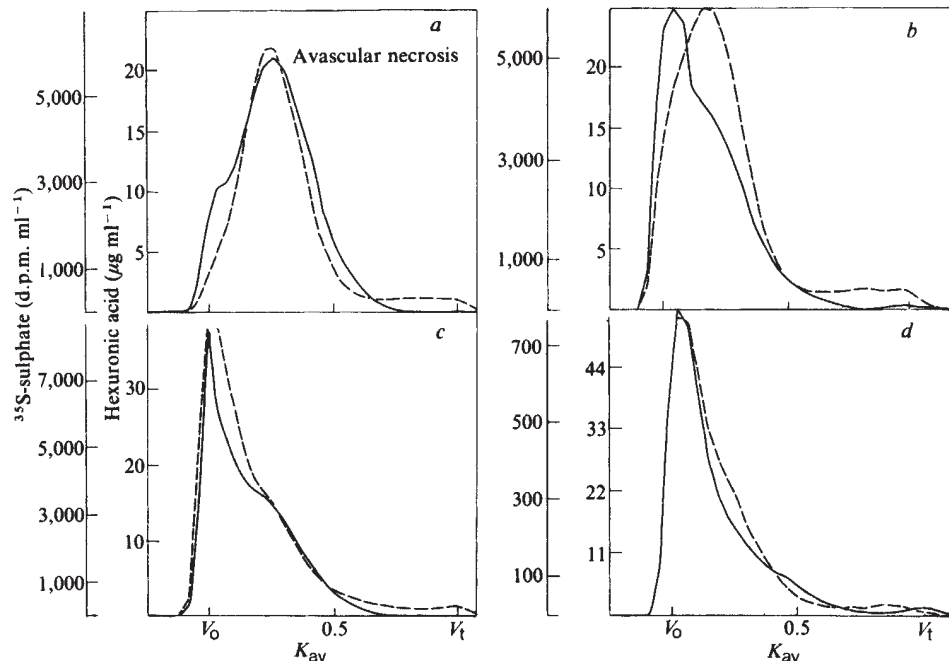
Departments of Orthopaedic Surgery and Biochemistry, University of Minnesota, Minneapolis, Minnesota 55455

Proteoglycans are major components of many extracellular matrices. In cartilage, they provide reversible resistance to compression and exist as molecules with molecular weights (MWs) of $1-3 \times 10^6$. There is a central protein core of MWs approximately 2×10^5 (refs 1, 2) with specialized subregions, one containing mainly the chondroitin sulphate chains³, another most of the keratan sulphate chains⁴, and a third is a largely globular structure interacting specifically with both hyaluronic acid⁵ and a link protein⁶ to form stable aggregate structures such as those identified in human articular cartilage⁷. In embryonic⁸ and tissue culture systems⁹, proteoglycans are isolated as aggregate structures in as little as 5-10 min after synthesis (sulphation) with no nonaggregating precursor detected. However, Heinegård and Hascall have characterized the small proportion of nonaggregating proteoglycan present in bovine nasal septum cartilage and found that it contained more peptide than the aggregating proteoglycan¹⁰. Work by Upholt *et al.*¹¹ has suggested that the MW of unprocessed protein core, synthesized by a wheat-germ translating system from chick sternal cartilage mRNA, is $\sim 340,000$, leaving open the possibility of intermediates. I report here the presence, in some human cartilages, of a proteoglycan population that initially will not aggregate with the hyaluronic acid but subsequently can be chased into aggregate.

Human articular cartilage samples were obtained as described previously¹² from normal femoral heads and osteoarthritic joints in the areas immediately adjacent to the osteoarthritic lesion and from kidney-transplant patients undergoing total hip arthroplasty for steroid-induced avascular necrosis. Samples were labelled *in vitro* in RPMI 1640 media as described previously (³⁵S-sulphate concentration of $100 \mu\text{Ci ml}^{-1}$) for 4 h (ref. 13) and then part of the sample was extracted with 4 M guanidine hydrochloride in the presence of protease inhibitors³. The remainder of the sample was transferred to nonradioactive media containing 0.5 mM sulphate, chased for 18 h, and subsequently extracted as the 4-h samples. The proteoglycan monomer was purified from the extract by a dissociative caesium chloride density-gradient procedure⁷. The proteoglycans were isolated in the bottom two-fifths or D1 fraction and were subsequently examined for incorporated isotope and the ability to interact with hyaluronic acid². The extracted residue was examined for nonextracted, incorporated isotope as described previously¹³.

Results indicated that both 4-h pulse and 18-h chase samples had the same total incorporation, so there was no incorporation of isotope during the chase period. Ninety-five per cent of the incorporated isotope was found in the bottom D1 or proteoglycan monomer fraction. The per cent extractable which varied from cartilage to cartilage (between 38 and 65%) was not different for the paired 4- and 18-h samples, indicating no significant change in the extractable pool size. There were three classes of response: ³⁵S-sulphate-labelled proteoglycan from normal cartilage ($n = 2$) interacted with hyaluronic acid to the same extent at both 4 and 18 h (60-70%). Proteoglycans from patients with avascular necrosis ($n = 3$) consistently would not interact with hyaluronic acid at 4 h but would interact at 18 h (Fig. 1b, c) with two additional samples having partial response at 4 h. The osteoarthritic cartilage ($n = 5$) gave intermediate

Fig. 1 The elution profiles for rat chondrosarcoma carrier proteoglycan hexuronic acid (—) and ^{35}S -sulphate (---) from Sepharose CL-2B for ^{35}S -sulphate-labelled proteoglycans isolated from avascular necrotic human articular cartilage. *a*, 4-h incubation sample with no added hyaluronic acid. *b*, 4-h incubation sample plus 4% (w/w) added hyaluronic acid. *c*, 18-h chase sample plus 4% added hyaluronic acid. *d*, Proteoglycan reisolated after tumour treatment plus 4% hyaluronic acid. For the 4-h sample, the D1-2 fraction contained 2.37×10^6 d.p.m. and represented 89% of the extracted d.p.m. and 46.7% of the total incorporated ^{35}S -sulphate. The 18-h chase D1-2 fraction contained 2.2×10^6 d.p.m. and represented 90% of the extracted d.p.m. and 50.8% of the total incorporated ^{35}S -sulphate. Of the tumour-treated D1-2 fraction, 97% of the incorporated ^{35}S -sulphate was in the D1-2 or bottom gradient fraction after the treatment, and 90% of the initially added isotope was recovered. The 18-h and tumour-treated controls with no added hyaluronic acid were identical to the 4-h control with no added hyaluronic acid. Carrier (2 mg) was added for each chromatographic run. The proteoglycan extracted from the tissue represented less than 5% of this value. The incubation with the chondrosarcoma included 0.5 g of finely isolated tumour that was finely minced and briefly washed with RPMI 1640 medium. The tumour was incubated for 4 h at 37 °C in 95% air–5% CO_2 with the labelled proteoglycan that had been dialysed into (3 ml) RPMI 1640. In controls, similarly treated labelled proteoglycans from the chondrosarcoma were recovered unchanged in these incubation conditions. The presence of 10% heat-inactivated fetal calf serum did not alter the results.

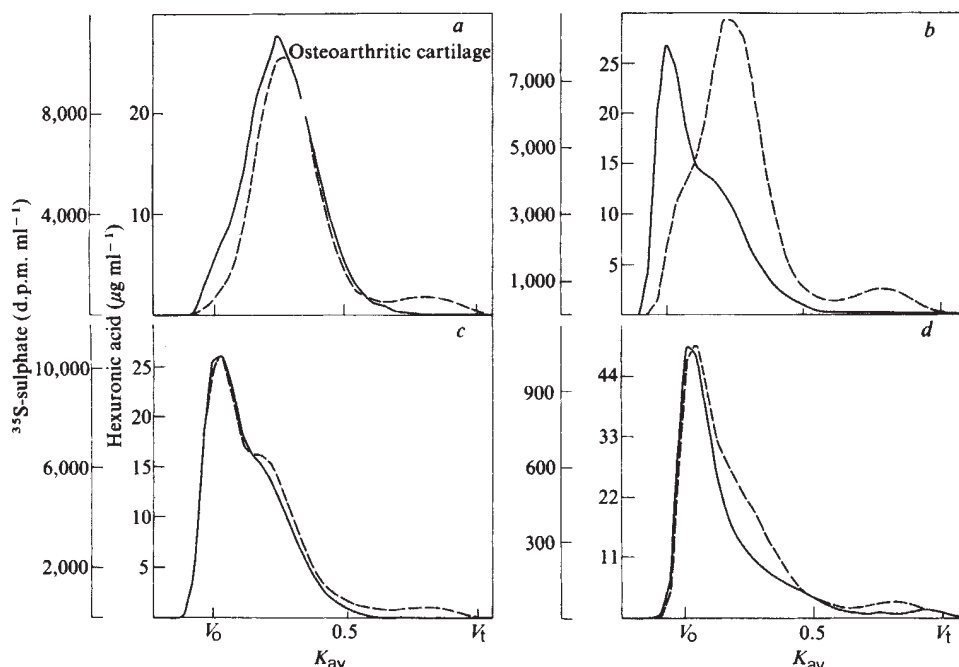


responses with some of the tissues, being unreactive at 4 h (Fig. 2b) and 70–80% reactive at 18 h (Fig. 2c). In some tissues, the proteoglycans were reactive to the extent of 20–30% at 4 h and were completely (60–70%) reactive at 18 h. When isolated ^{35}S -sulphate-labelled proteoglycan samples that were non-interactive at 4 h, were treated in the presence of a mince of rat chondrosarcoma² for 4 h, reisolated into a dissociative gradient and re-treated with hyaluronic acid, the samples then formed 60–70% aggregate (Figs 1d, 2d). Controls, in which the tumour was boiled before incubation, showed no conversion to a

hyaluronic interactive form, suggesting a required heat-labile component for conversion.

I therefore suggest that there is a delayed conversion from a hyaluronate-noninteractive precursor to a form that can interact with hyaluronic acid in some osteoarthritic and avascular human cartilages. At present, there is no evidence that this is a classical conversion of a proform to an interactive form or whether there may be a subtle alteration in the structure, such as the slow formation of the correct disulphide bonds in these disease-affected human cartilages. Furthermore, these results may be of

Fig. 2 The elution profiles for rat chondrosarcoma carrier hexuronic acid (—) and ^{35}S -sulphate (---) from Sepharose CL-2B for samples of proteoglycans isolated from osteoarthritic cartilage. *a*, 4-h incubation sample with no added hyaluronic acid. *b*, 4-h incubation sample plus 4% (w/w) added hyaluronic acid. *c*, 18-h chase sample plus 4% hyaluronic acid. *d*, Proteoglycan reisolated after treatment with chondrosarcoma plus 4% hyaluronic acid. For the 4-h sample, the D1-2 fraction contained 1.79×10^6 d.p.m. and represented 87% of the extracted d.p.m. and 53% of the total incorporated isotope. The 18-h chase D1-2 fraction contained 1.89×10^6 d.p.m. and represented 90% of the extracted d.p.m. and 69% of the total incorporated isotope. Of the tumour-treated D1-2 fraction, 97% of the ^{35}S -sulphate incorporation was in the D1-2 or bottom gradient fraction after the treatment and over 90% of the initially added isotope was recovered. The 18-h and tumour-treated controls with no added hyaluronic acid were identical to the 4-h control with no added hyaluronic acid.



more general application, as the hyaluronic acid-dependent proteoglycan aggregate has recently been reported in other systems^{13,14}.

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- Hascall, V. C. & Riolo, R. L. *J. biol. Chem.* **247**, 4529–4538 (1972).
- Oegema, T. R. Jr, Brown, M. & Dziewiatkowski, D. D. *J. biol. Chem.* **252**, 6470–6477 (1977).
- Heinegard, D. *J. biol. Chem.* **252**, 1980–1989 (1977).
- Heinegard, D. & Axelsson, I. *J. biol. Chem.* **252**, 1971–1979 (1977).
- Hascall, V. C. *J. supramolec. Struct.* **7**, 101–120 (1977).
- Baker, J. R. & Caterson, B. *J. biol. Chem.* **254**, 2387–2393 (1979).
- Bayliss, M. T. & Ali, S. Y. *Biochem. J.* **176**, 683–693 (1978).
- DeLuca, S., Caplan, A. I. & Hascall, V. C. *J. biol. Chem.* **253**, 4713–4720 (1978).
- Kimura, H. H., Hardingham, T. E., Hascall, V. C. & Solursh, M. *J. biol. Chem.* **254**, 2600–2609 (1979).
- Heinegard, D. & Hascall, V. C. *J. biol. Chem.* **254**, 927–934 (1979).
- Upholt, W. B., Vertel, B. M. & Dorfman, A. *Proc. natn. Acad. Sci. U.S.A.* **79**, 4847–4851 (1979).
- Thompson, R. C. Jr & Oegema, T. R. Jr *J. Bone Jt Surg.* **61**, 407–416 (1979).
- Oegema, T. R. Jr, Bradford, D. S. & Cooper, K. C. *J. biol. Chem.* **254**, 10579–10581 (1979).
- Oegema, T. R. Jr, Hascall, V. C. & Eisenstein R. *J. biol. Chem.* **254**, 1312–1318 (1979).

Use of aequorin to study excitation–contraction coupling in mammalian smooth muscle

Ian R. Neering* & Kathleen G. Morgan

Departments of Pharmacology and Physiology and Biophysics, Mayo Foundation, Rochester, Minnesota 55901

Considerable information is available for intact skeletal and cardiac muscle cells on the role of changes in intracellular Ca^{2+} (Ca_i^{2+}) levels during excitation–contraction coupling^{1–3}. Ca_i^{2+} levels seem to be similarly important in smooth muscle^{4,5}. However, only two published reports describe the use of indicators to directly measure $[\text{Ca}_i^{2+}]$ in intact smooth muscle cells. One described the use of murexide in molluscan smooth muscle⁶, the other the use of aequorin in single amphibian cells⁷. Their results differed: very long calcium transients were described for the molluscan muscle, compared with very brief ones for the amphibian cells. We present here the first description of the relationship between calcium transients and contraction of whole, mammalian smooth muscle injected with aequorin. The Ca^{2+} transient we observed has a slow rise, a long duration, and there was no delay between the peak of the Ca^{2+} transient and the onset of force. This contrasts with the amphibian findings but resembles the molluscan data. We observed a delay between the onset of the increase in $[\text{Ca}_i^{2+}]$ and the onset of the rise in tension which might be explained, at least in part, by the presence of a threshold $[\text{Ca}_i^{2+}]$ for concentration.

Strips of dog antral circular smooth muscle were prepared as described previously⁸. In each preparation aequorin^{9,10}, dissolved in 150 mM KCl, 5 mM HEPES, pH 8.0, was pressure-injected through glass micropipettes into 20–60 cells over an approximately 1-mm² area. Light from the aequorin-injected area was detected with an EM1 9635A photomultiplier tube¹. Figure 1a shows simultaneously recorded light and tension signals in the preparation which gave the largest light response. Both light and tension transients were reproducibly recorded in excess of 128 responses occurring over more than 2 h. This indicates that significant consumption of aequorin was not occurring over this time period. Figure 1b, c shows averaged signals giving a clearer indication of the relationship between light and tension. The onset of the stimulus was coincident with an immediate rapid increase of luminescence (Fig. 1c,

arrowhead) (time to peak 3–4 ms) lasting for the duration of the stimulus and decaying with the same time course as the rise. The short-duration light response was followed by a much slower increase in luminescence with a time to peak of 200 ms (range in five preparations 200–800 ms) and a half decay time of 2 s (range 2–2.5 s). The short-duration response was particularly sensitive to stimulus intensity. At low intensities it was virtually absent, whereas at high intensities light saturated the recording system. At the same, low intensities, the longer-duration light signal and the contraction persisted and an actual potential was still triggered. We think that the short-duration light signal is probably associated with an abnormal membrane response, which has been postulated to explain similar transients in amphibian smooth muscle⁷. Action potentials in canine gastric smooth muscle are often 10 s in duration and seem to involve a Ca^{2+} current⁸. Thus, a component of the slower light (Ca_i^{2+}) transient may occur as a result of Ca^{2+} influx during the action potential; however, intracellular release of calcium may also be involved.

It is interesting to compare the responses obtained here with those obtained in other muscles. In amphibian skeletal muscle the delay between onset of the light signal and onset of tension is 5–10 ms at 10 °C (ref. 1); in mammalian Purkinje fibres it is 18–20 ms at 35 °C (ref. 2); in amphibian smooth muscle the delay is greater than 200 ms at 25 °C (ref. 7), whereas for the slow response in our mammalian smooth muscle preparation at 35 °C the delay is 100–160 ms. The value of this latency was not reported for molluscan smooth muscle⁶. Thus, in this respect, mammalian smooth muscle seems to resemble amphibian smooth muscle more closely than the other muscle types. However, the time to peak amplitude of the light responses in mammalian smooth muscle is much greater than in amphibian muscle. Amphibian smooth muscle has a time to peak of only 3 or 4 ms and the light transient is virtually over well before tension development. In contrast, this mammalian smooth muscle has a time to peak of 200–800 ms and a peak light emission coincident with the development of tension. The time to peak for molluscan smooth muscle is similarly long, reaching 1 s or more and corresponding with the development of tension. For comparison, skeletal and cardiac muscles have times to peak of 4 and 8–20 ms, respectively, and the peak light emission occurs during the phase of tension development.

The observation that light emission and hence $[\text{Ca}_i^{2+}]$ of mammalian smooth muscle rises for a period of more than 100 ms before there is any detectable increase in tension may be interpreted in several ways; either there are relatively slow steps between the elevation of Ca_i^{2+} and tension development, or $[\text{Ca}_i^{2+}]$ is below a threshold concentration necessary for the development of force and some time would be taken in reaching this level, or both of these alternatives occur. The second possibility is supported by the report that this tissue has a voltage threshold for contraction some 30 mV positive to its resting potential^{11,12}. To test this possibility and also to determine whether $[\text{Ca}_i^{2+}]$ is voltage dependent, we recorded calcium levels in depolarized preparations. Figure 2a shows the changes in light and tension with time when an antral strip was depolarized with a solution containing 90 mM K^+ . This preparation had a 'resting glow' detectable signal averaging. In 5 of 13 preparations demonstrating such a resting glow, there was a decrease in light intensity during the first minute of exposure, however, no regular pattern in this occurrence was noticed. Concentrations of K^+ of 18–27 mM depolarized the membrane by approximately 15–30 mV (refs 11, 12) and caused increases in light but no increase in tension. Higher levels of K^+ caused concentration-related increases in light and tension. Figure 2b shows typical plots of both light and tension as a function of $[\text{K}^+]$ while in Fig. 2c these data are presented as a function of light against tension. Clearly light increased as the $[\text{K}^+]$ was raised to 27 mM but there was negligible increase of tension up to this level. Microscopic examination of the tissue in these low $[\text{K}^+]$ solutions failed to reveal any evidence of internal shortening. Thus, $[\text{Ca}_i^{2+}]$ was increasing at values of membrane potential

* Present address: Department of Physiology and Pharmacology, University of New South Wales, Box 1, PO Kensington, Sydney, Australia.

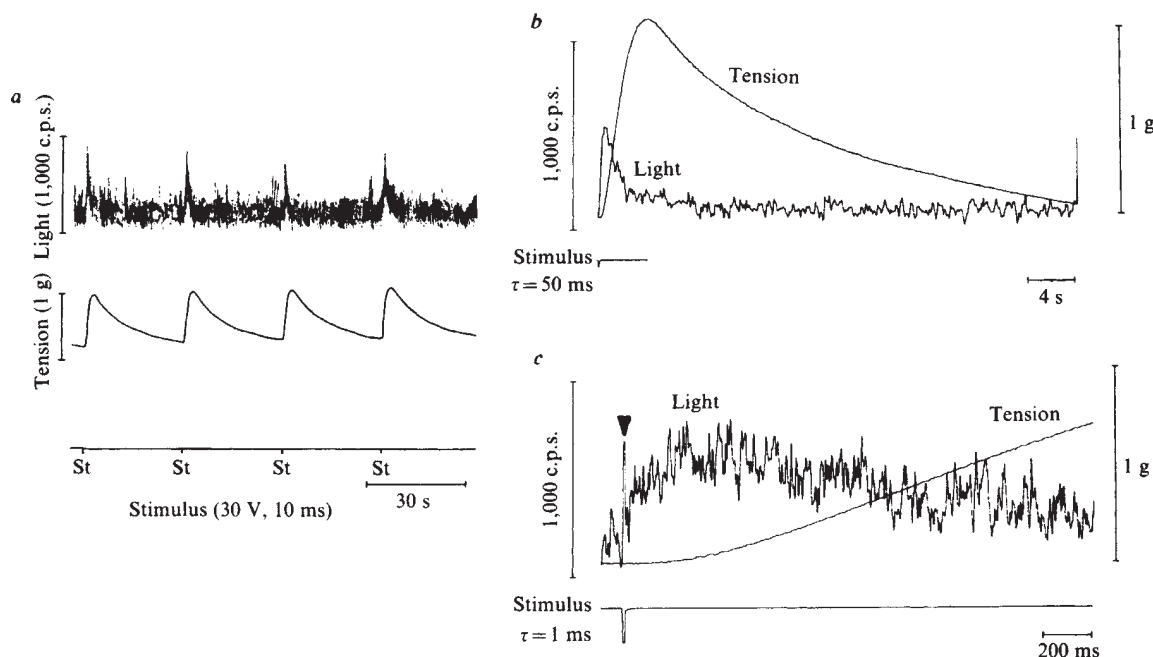


Fig. 1 *a*, Simultaneous recordings of light (upper trace) and force of isometric contraction. Light is measured in counts per s from a photon-counting system and force is measured in grammes from a Satham force transducer with a natural frequency of 250 Hz. Individual light and tension responses elicited by 10-ms, 50-V stimuli are shown. The preparation was stimulated through a pair of Pt wires 15 mm apart and parallel to the axis of the circular fibres. Stimulus voltage was chosen to be supramaximal for contraction. Electrical stimuli of these parameters triggered action potentials in this tissue. About 40 cells in this preparation were injected with aequorin. The injection of aequorin had no detectable effect on membrane potential or action potential generation in this spontaneously active tissue. *b*, 32 simultaneous light and tension signals have been averaged to obtain this record. The light signal has been filtered with a 50-ms time constant. *c*, Expanded time scale of the data shown in *b*; recorded with a 1-ms time constant. Arrowhead denotes the brief response associated with stimulus.

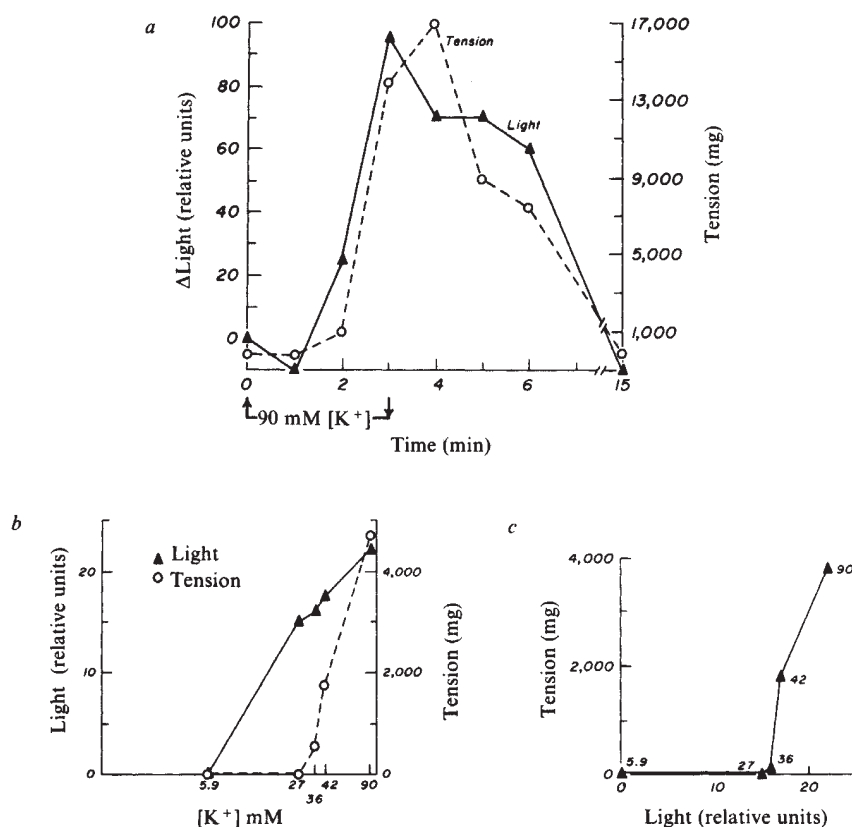


Fig. 2 *a*, Effect of 90 mM K⁺ on light emission and isometric tension development. The high [K⁺] solution was substituted for normal Krebs solution at 0 time. ○, Tension; △, light; each data point represents the average of 60, 1-s segments of both light and tension recordings before, during and after exposure to K⁺. High [K⁺] was washed out in this experiment before steady state was established. *b*, Relationship between [K⁺] and light and tension. All data points are from the same preparation, and taken for steady-state responses to each solution. 5.9 mM is the concentration of K⁺ in the normal Krebs. *c*, Relationship between light and tension induced by different levels of [K⁺]. This graph shows the same data as *b*. The number beside each symbol represents the [K⁺]. Solutions of high [K⁺] were prepared by equimolar substitution of K⁺ for Na⁺. Temperature in all experiments was 32–35 °C (constant in any one experiment).

subthreshold for contraction. This observation supports the contention that resting calcium levels in this tissue are somewhat below a calcium threshold. As this tissue does not normally demonstrate active tone, it may be that such tissues are distinguished from tissues with tonic activity by the level of resting intracellular free calcium concentration.

Whether this 'calcium threshold' is the threshold level of Ca^{2+} which activates the myofilaments or that which causes sufficient activation to take up the compliance of the tissue was not determined. Note, however, that with smooth muscle from the fundal region of the stomach¹¹, and using the same apparatus for recording contractile tension, significant tension development is detected with as low a $[\text{K}^+]$ as 12 mM.

Recently, it has been suggested that the action of Ca^{2+} on the contractile system of smooth muscle is not direct but, rather, that the Ca^{2+} activates a protein kinase which phosphorylates the

myosin and stimulates myosin's interaction with actin¹³⁻¹⁵. This possibility has been used to explain the long delay between the calcium transient and contraction in amphibian smooth muscle⁷. As we do not observe any similar delay in our mammalian smooth muscle preparation, we conclude that such phosphorylation either is not required in mammalian smooth muscle at 35 °C or that it is considerably more rapid than in amphibian smooth muscle at 24 °C. We favour the latter interpretation because the long time course of dephosphorylation¹⁶ could explain the lag between the return of $[\text{Ca}^{2+}]$ to basal levels and the relaxation of the muscle.

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1. Blinks, J. R., Rüdel, R. & Taylor, S. R. *J. Physiol., Lond.* **277**, 291-323 (1978).
2. Wier, W. G. *Science* **207**, 1085-1087 (1980).
3. Allen, D. G. & Blinks, J. R. *Nature* **273**, 509-513 (1978).
4. Hartshorne, D. J. in *Handbook of Physiology, The Cardiovascular System* Vol. II, 93-120 (Am. Physiol. Soc., Bethesda, Maryland, 1980).
5. Endo, M., Kitazawa, T., Yagi, S., Iino, M. & Kakuta, Y. in *Excitation-Contraction Coupling in Smooth Muscle* (eds Casteels, R. et al.) 199-210 (Elsevier, Amsterdam, 1977).
6. Kometani, K. & Sugi, H. *Experientia* **34**, 1469-1470 (1978).
7. Fay, F. S., Shlevin, H. H., Granger, W. C. & Taylor, S. R. *Nature* **280**, 506-508 (1979).
8. El-Sharkawy, T. Y., Morgan, K. G. & Szurszewski, J. H. *J. Physiol., Lond.* **279**, 291-307 (1978).

9. Blinks, J. R., Prendergast, F. G. & Allen, D. G. *Pharmac. Rev.* **28**, 1-93 (1976).
10. Blinks, J. R. et al. *Meth. Enzym.* **57**, 292-328 (1978).
11. Morgan, K. G., Muir, T. C. & Szurszewski, J. H. *J. Physiol., Lond.* (in the press).
12. Morgan, K. G. & Szurszewski, J. H. *J. Physiol., Lond.* **301**, 229-242 (1980).
13. Chacko, S., Conti, M. A. & Adelstein, R. S. *Proc. natn. Acad. Sci. U.S.A.* **74**, 129-133 (1977).
14. Bremel, R. D., Sobieszek, A. & Small, J. V. in *The Biochemistry of Smooth Muscle* (ed. Stephens, N. L.) 533-549 (University Park Press, Baltimore, 1977).
15. Aksoy, M. O., Williams, D., Sharkey, E. M. & Hartshorne, D. J. *Biochem. biophys. Res. Commun.* **69**, 35-41 (1976).
16. Aksoy, M. O., Dillon, P. F. & Murphy, *Fedn Proc.* **39**, 2042-2044 (1980).

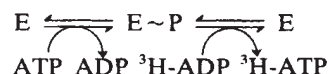
External Na dependence of ouabain-sensitive ATP:ADP exchange initiated by photolysis of intracellular caged-ATP in human red cell ghosts

Jack H. Kaplan & Richard J. Hollis

Department of Physiology and Biophysics, University of Iowa, Iowa City, Iowa 52242

Coupled active transport of Na^+ and K^+ across cellular plasma membranes is mediated by $(\text{Na}^+ + \text{K}^+)\text{-stimulated Mg}^{2+}$ -dependent ATPase. Active cation transport by this Na pump involves a cyclic Na-dependent phosphorylation of the enzyme by intracellular ATP and hydrolytic dephosphorylation of the phosphoenzyme, stimulated by K^+ (ref. 1). In human red blood cells², skeletal muscle³ and squid axons^{3,4}, replacement of extracellular K by Na results in a ouabain-sensitive efflux of Na coupled to an influx of extracellular Na. There is apparently no net Na movement⁵ nor net hydrolysis of ATP⁶. The rate of Na:Na exchange is stimulated by increased levels of ADP⁷ and exchange transport is not observed in cells totally depleted of intracellular ATP⁸. These characteristics suggest that the biochemical mechanism underlying the Na exchange mode of the Na pump involves phosphorylation of the enzyme by ATP (which requires intracellular Na) followed by its dephosphorylation by ADP. Such a reaction has been observed in partially purified $(\text{Na}^+ + \text{K}^+)\text{ATPase}$ from a variety of sources⁹⁻¹¹ and its dependence on Na concentration has been described^{12,13} (although not previously for the red cell enzyme). In the present work, intracellular ATP:ADP exchange reaction was initiated by photoreleased ATP following brief irradiation at 350 nm of ghosts containing caged-ATP¹⁴. The ouabain-sensitive component of the ensuing ATP:ADP exchange reaction shows a biphasic response to extracellular Na. External Na in the range 0-10 mM has an inhibitory effect whilst increasing concentrations beyond this range stimulate the rate of exchange in a roughly linear fashion up to 100 mM Na. These results represent the first direct demonstration of the sidedness of the effects of Na on this partial sequence in the overall enzyme cycle and bear a qualitative resemblance to the Na effects on the Na-ATPase which occur in the absence of intracellular ADP in human red blood cells¹⁵.

The biochemical reaction of interest in the present context may be represented as,



and can be measured as the ouabain-sensitive rate of production of $^3\text{H-ATP}$ from $^3\text{H-ADP}$. Although the availability of resealed erythrocyte ghosts has made possible the independent manipulation of intracellular and extracellular cation concentrations, in order to measure the ATP:ADP exchange reaction, three major experimental difficulties had to be overcome: (1) high cytoplasmic adenylate kinase activities (approximately 5 mol per litre of cells per hour); (2) high cytoplasmic nucleoside diphosphokinase (NPDK) activities; (3) endogenous ATPase activities. Furthermore, the preparation of resealed ghosts requires a lengthy incubation procedure for resealing. Since the reaction of interest involves isotopic exchange in the intracellular compartment a recycling system is not appropriate and to measure initial rates of the unidirectional reaction it is necessary that the reaction be initiated after ghost preparation is completed.

Following the lead of Cavieres and Glynn⁸ we prepared resealed ghosts with a large dilution of cellular contents and incorporated inside the ghosts the competitive inhibitor of adenylate kinase, diadenosine pentaphosphate (Ap_5A) (ref. 16). Using a gel filtration column for cell lysis and cytoplasmic dilution¹⁷ and incorporating 50 μM Ap_5A into the ghosts we have been able to prepare ghosts containing up to 250 μM $^3\text{H-ADP}$ which after incubation at 37 °C for 2 h contained $^3\text{H-AMP}$ at levels that were no higher than originally present in the $^3\text{H-ADP}$ as a contaminant (about 4%). The presence of high cytoplasmic NPDK activities in human erythrocytes is well documented¹⁸ (approximately 5 mol per litre cells per h) and we find that sufficient activity is present even in 300-fold diluted hemolysates to generate micromolar levels of ATP from micromolar ADP and millimolar UTP within several minutes. The enzyme performs the same reaction as the one of interest mediated by the Na pump, that is, the production of $^3\text{H-ATP}$ from $^3\text{H-ADP}$ via the dephosphorylation of a phosphoenzyme intermediate. The brain NPDK enzyme is inhibited by Cibacron blue F3GA (J. B. Robinson and E. C. Stellwagen, personal

communication). Unfortunately, although this dye is also a very potent inhibitor of the cytoplasmic red cell enzyme, it partitions predominantly into cell membranes. The inhibition of cytoplasmic NPDK activity in red cell haemolysates containing Cibacron blue is removed on the addition of red cell membranes (J.H.K. and R.J.H., unpublished data). Amongst the class of dyes which inhibit nucleotide-requiring enzymes¹⁹, Trypan blue (CI 23850) was found to inhibit cytoplasmic NPDK activities in the absence and presence of red cell membranes and in resealed red cell ghosts, without affecting $(\text{Na}^+ + \text{K}^+)\text{ATPase}$ activities.

To avoid the problems associated with endogenous ATPase activity during ghost incubations at 37 °C (which are required to reseal the ghosts) and also in order to initiate the exchange reaction, we have incorporated caged-ATP into the ghosts. Caged-ATP, $\text{P}^3\text{-1-(2-nitro)phenylethyl-ATP}$, is an ATP analogue in which the terminal phosphate of ATP is esterified by a 2-nitrobenzyl moiety. Caged-ATP is resistant to ATPase

activity because of the esterification of the terminal phosphate residue and is unable to perform ATP-dependent reactions which require phosphorylation. The 2-nitrobenzyl residue is cleaved from the rest of the molecule by irradiation at 350 nm, releasing ATP¹⁴. We have used caged-ATP as a stable source of ATP that will not undergo reaction during ghost preparation but which subsequently can be 'activated' by the photorelease procedure.

Figure 1 illustrates the Na dependence of ATP:ADP exchange rates in porous red cell membranes. The curve is very similar to that seen using partially purified enzyme from pig kidney outer medulla¹⁰ or dog kidney outer medulla (J.H.K. and M. D. Mone, unpublished), although the specific activity in red cell membranes is some 2–3 orders of magnitude lower than the kidney preparations.

In order to decide which of the Na effects are mediated at extracellular sites, resealed red cell ghosts were prepared containing, MgSO_4 , NaCl, choline Cl, the kinase inhibitors,

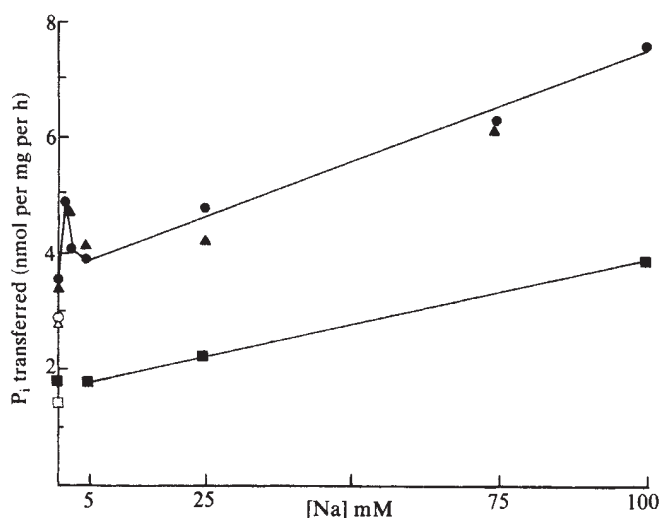


Fig. 1 The Na dependence of ATP:ADP exchange in porous red cell membranes. Haemoglobin-free membranes were prepared essentially according to the method of Fairbanks *et al.*²¹ by lysis of cells at 0 °C in 20 vol of 5 mM sodium phosphate pH 8. Before lysis the cells were washed in 150 mM NaHCO_3 pH 8.2 to facilitate haemoglobin release²². Until use, the white membranes were stored at -20 °C. Thawed membranes were suspended in 5 mM EDTA, 10 mM HEPES pH 7.5 (at 0 °C), incubated at room temperature for 10 min and pelleted (35,000 g, 15 min). The pellet was then resuspended in 150 mM choline chloride, 10 mM HEPES pH 7.6 in 50 μl aliquots at a protein concentration of approximately 3 mg ml^{-1} . The reactions were then carried out at 23 °C following the addition of an equal volume of buffered solution containing (final concentration) NaCl + choline chloride to 150 mM, 10 mM MgCl_2 , 25 μM $^3\text{H-ADP}$, 25 μM ATP, 100 μM Ap_5A and 10 mM HEPES pH 7.6. The reaction was started by the addition of the nucleotides and sampled by quenching in ice-cold 10% trichloroacetic acid at 0.25, 5.25 and 10.25 min. The samples were then frozen before processing. The thawed samples were centrifuged for 2 min at 10,000g in an Eppendorf microfuge, to pellet the denatured membranes, and samples of the supernatant applied to polyethyleneimine thin layer chromatography plates. The plates were dried and samples of a solution containing 4 mM each of ATP, ADP and AMP applied on top of the experimental samples. The plates were then developed in 0.4 M K phosphate, pH 3.5 and the regions containing ATP, ADP and AMP, visualized using an UV lamp, cut out and placed in scintillation vials. Following the addition of HCl (1 M, 0.5 ml) the vials were placed in an oven at 120 °C for 30 min. After cooling, scintillation fluid was added and then radioactivity co-migrating with each nucleotide counted. Recovery of radioactivity using this procedure was always between 97% and 103% of the amount applied to the chromatography plate. The rates were linear over the time period of the reaction and the initial rate calculated. The curves shown depict the dependence of the exchange rate on Na concentration in experiments using three different preparations of membranes. The open symbols are rates obtained in the absence of Na with 10^{-4} M ouabain in the medium. In the lowest curve Na concentrations between 0 and 5 mM were not used. Although the dependence of rate upon Na concentration was the same in different membrane preparations, the absolute rates did show some variation in different preparations.

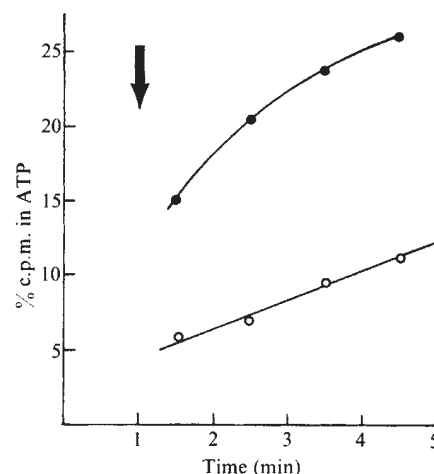


Fig. 2 Photolytic initiation of the ATP:ADP exchange reaction in resealed red cell ghosts. Freshly drawn blood was collected in heparinized tubes and the red cells washed three times in 150 mM choline chloride, 10 mM HEPES pH 7.6 by centrifugation (14,000g, 5 min), removal of supernatant and resuspension. After the second resuspension the cells were incubated at room temperature for 10 min to ensure completion of pH equilibration. A 10% (by vol) suspension of the cells in the choline buffer was cooled to 0 °C. The suspension was then introduced into the top of a jacketed column of Biorad A50m gel (35 cm $\times$ 5.0 cm) maintained at -1 °C. The column had previously been prepared so that the lower half was equilibrated with 15 mM PIPES, 0.1 mM EDTA pH 6.0, the middle four-tenths of column volume with 20 mM HEPES, pH 7.5 and the upper one-tenth with 150 mM choline Cl, 10 mM HEPES pH 7.6. After introducing the cells into the column they were eluted with buffered choline Cl. The eluate was collected in ice-cold tubes as soon as turbidity was observed in the eluate leaving the column. At this stage the red band of haemoglobin was approximately half-way down the column. The fractions containing membranes were bulked and collected by centrifugation at 0 °C, 31,000g for 15 min. The supernatant was removed and ice-cold reversal solution containing choline Cl + NaCl (150 mM), 50 μM Trypan blue, 100 μM Ap_5A , 10 μM MgSO_4 , 50 μM caged-ATP, 25 μM $^3\text{H-ADP}$, 10 mM HEPES pH 7.6 was added to the pale pink pellet. After resuspension and incubation in ice for 5 min, the membranes were transferred to a water bath at 37 °C for a further 45 min. The resealed ghosts were then washed three times in 150 mM choline Cl, 10 mM HEPES pH 7.6 by centrifugation (31,000g, 5 min) and resuspension. The ghosts were packed after the final wash (31,000g, 15 min) and resuspended in 10% suspension (by vol) in 100 mM NaCl, 50 mM choline Cl, 10 mM HEPES pH 7.6. Aliquots (200 μl) of the suspension were photolysed in 1 mm path length cuvettes as previously described¹⁴ and at suitable time intervals samples (20 μl) were added to ice-cold 10% trichloroacetic acid (20 μl) and rapidly mixed. Duplicate samples were added to distilled water (200 μl) and placed in a boiling water bath for 1 min. The boiled samples were then cooled and assayed for ATP, ADP and AMP contents using a modification of the procedure of Kimmich *et al.*²⁰. The samples in trichloroacetic acid were treated as described in the legend to Fig. 1. The filled symbols indicate the rate of appearance of radioactivity in intracellular ATP, the open symbols the rate in the presence of 10^{-4} M ouabain in the external medium. During the time of reaction ATP hydrolysis was less than 5%. The reaction was initiated by photolysis at zero time, the vertical arrow indicates when photolysis was stopped. In unphotolysed ghosts less than 0.04% of the total radioactivity migrated with ATP.

^3H -ADP and caged-ATP and photolysed to initiate the exchange reaction. A representative time course of the appearance of ^3H -ATP in such an experiment is shown in Fig. 2. It is clear that a major component of the total exchange reaction is ouabain-sensitive, that is, mediated by the Na pump. Figure 3 shows the data from experiments in which external Na has been systematically varied. In each experiment the rate of ATP:ADP exchange was measured in aliquots of the ghosts suspended at various external Na concentrations. The curves depict the variation of the ATP:ADP exchange rate as a function of the external Na concentration at constant internal Na.

Thus Na pump-mediated ATP:ADP exchange in resealed human red cell ghosts shows a biphasic dependence on extracellular Na concentration. These observations enable us to identify unambiguously each of the three limbs of the Na activation curve in Fig. 1 with intracellular or extracellular sites for Na. At least three classes of sites are observed on the porous membranes (Fig. 1) and of these, the two of lower affinity can now be localized to the extracellular surface of the cell membrane. The higher-affinity extracellular site inhibits the ATP:ADP exchange reaction in the range 0–10 mM Na, the lower affinity site stimulates the reaction in an approximately linear fashion up to 100 mM Na, and thus saturates well beyond the physiological range. The highest affinity Na sites are intracellular and presumably support enzyme phosphorylation. It has been suggested that the stimulation of ATP:ADP exchange reaction rate seen at high Na levels with porous enzyme preparations is due to an increase in the steady-state level of ADP-sensitive phosphoenzyme¹³. Our data extend the earlier observations to include red cell membranes and further specify that the Na sites which are involved in this stimulation are sites for external Na.

The effects of external Na on ATP:ADP exchange described here parallel the dependence of Na:Na exchange in red blood cells on external Na concentration⁵. The question of the relative rates of these reactions has not been directly addressed in the present studies. It would be interesting to know how many Na ions are transported across the membrane each time the phosphorylated pump protein is dephosphorylated by ADP. Although Na entry may require the dephosphorylation step⁷ the converse is apparently not true. When external Na is zero and Na entry cannot take place ATP:ADP exchange does occur (Fig. 3). Under these conditions it is not known whether a Na efflux occurs, whether internally bound Na is discharged into the cytosol or whether internal Na remains bound to the enzyme.

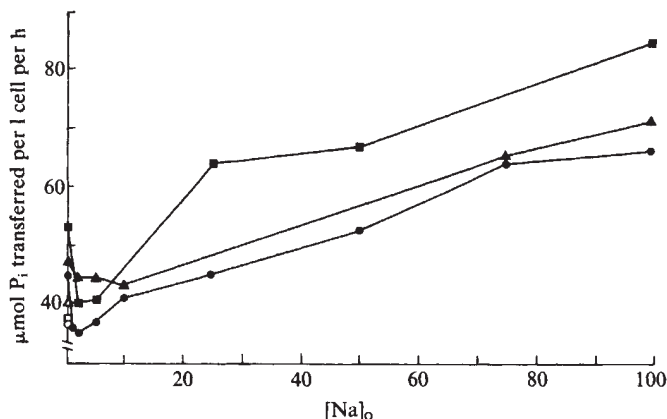


Fig. 3 Dependence of the rate of ATP:ADP exchange on extracellular Na. The procedures used to prepare the ghosts and determine the rate of ATP:ADP exchange were as described in the legends to Figs 1 and 2. The ghosts were suspended in media containing varying Na concentrations before photolysis, maintaining the sum NaCl+choline Cl at 150 mM. The three curves illustrate data from three different batches of resealed ghosts. The open symbols show the rate of ATP:ADP exchange in the absence of extracellular Na and in the presence of 10^{-4} M ouabain. Intracellular Na was determined by flame photometry and was between 11 mM and 16 mM in these experiments.

The determination of the relationship between the rates of isotopic Na movements and ATP:ADP exchange awaits their measurement under varied identical conditions in the same preparation.

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- Glynn, I. M. & Kariish, S. J. D. *A. Rev. Physiol.* **37**, 13–55 (1975).
- Garrahan, P. J. & Glynn, I. M. *J. Physiol., Lond.* **192**, 189–216 (1967).
- Kennedy, B. G. & DeWeer, P. *J. gen. Physiol.* **68**, 405–420 (1976).
- DeWeer, P. *Nature* **219**, 730–731 (1968); **226**, 1251–1252 (1970).
- Garrahan, P. J. & Glynn, I. M. *J. Physiol., Lond.* **192**, 159–174 (1967).
- Garrahan, P. J. & Glynn, I. M. *J. Physiol., Lond.* **192**, 217–235 (1967).
- Glynn, I. M. & Hoffman, J. F. *J. Physiol., Lond.* **218**, 239–256 (1971).
- Cavieser, J. D. & Glynn, I. M. *J. Physiol., Lond.* **297**, 637–645 (1979).
- Fahn, S., Koval, G. J. & Albers, R. W. *J. biol. Chem.* **241**, 1882–1889 (1966).
- Stahl, W. L. *J. Neurochem.* **15**, 499–509; 511–518 (1968).
- Banerjee, S. P. & Wong, S. M. E. *J. biol. Chem.* **247**, 5409–5413 (1972).
- Wildes, R. A., Evans, H. J. & Chiu, J. *Biochim. biophys. Acta* **307**, 162–168 (1973).
- Beauge, L. A. & Glynn, I. M. *J. Physiol., Lond.* **289**, 17–31 (1979).
- Kaplan, J. H., Forbush III, B. & Hoffman, J. F. *Biochemistry* **17**, 1929–1935 (1978).
- Glynn, I. M. & Kariish, S. J. D. *J. Physiol., Lond.* **256**, 465–496 (1976).
- Lienhard, G. E. & Secemski, I. I. *J. biol. Chem.* **248**, 1121–1123 (1973).
- Wood, P. G. *Fedn. Proc.* **34**, 249 (1975).
- Parks, R. E. Jr. & Agarwal, R. P. *Enzymes* **8**, 307–333 (1973).
- Stellwagen, E. C. *Acc. Chem. Res.* **10**, 92–98 (1977).
- Kimmich, G. A., Randles, J. & Brand, J. S. *Anal. Biochem.* **69**, 187–209 (1975).
- Fairbanks, G., Steck, T. L. & Wallach, D. F. H. *Biochemistry* **10**, 2606–2616 (1971).
- Jennings, M. L. & Passow, H. *Biochim. biophys. Acta* **554**, 498–519 (1979).
- Caldwell, P. C., Hodgkin, A. L., Keynes, R. D. & Shaw, T. I. *J. Physiol., Lond.* **152**, 561–690 (1960).

Potent carcinogenicity of nitrosodiethanolamine in rats

W. Lijinsky, M. D. Reuber & W. B. Manning

Chemical Carcinogenesis Program, NCI Frederick Cancer Research Center, Frederick, Maryland 21701

Nitrosodiethanolamine is found in synthetic cutting oils and in many cosmetic preparations and is probably the *N*-nitroso compound to which human exposure is greatest^{1–3}. It is formed by reaction of the commonly used amines diethanolamine and triethanolamine⁴ with nitrosating agents. An assessment of the possible risk in human exposure to nitrosodiethanolamine must be based on sound chronic toxicity data. A previously published chronic test of this compound in rats has shown it to induce liver tumours after very high oral doses⁵, and tumours of the nasal cavity after administration of high repeated doses to Syrian hamsters by subcutaneous injection⁶. To improve our understanding of the carcinogenic potency of nitrosodiethanolamine, we undertook a more extensive study, in which the compound was administered at concentrations ranging from 3,900 to 31,250 parts per million (p.p.m.) in drinking water, to groups of rats for about 6 months. We report here that when the animals were killed, all bore hepatocellular carcinomas, many of which metastasized at the higher doses, indicating that nitrosodiethanolamine is a carcinogen of considerable potency in the rat. However, it is inactive or very weakly active in short-term tests, such as the *Salmonella* mutagenesis test developed by Ames⁷.

Sixty male and sixty female Fischer 344 rats of the colony of the Frederick Cancer Research Center, bred and raised in SPF conditions, were housed in plastic cages with wire-mesh bottoms (five rats per cage). The animals were 6–7 weeks old at the beginning of the experiment, and were randomized into (for

Table 1 Treatment of F 344 rats with solutions of nitrosodiethanolamine (NDELA) in drinking water

Group	NDELA concentration (p.p.m.)	No. of animals treated		No. of animals with liver tumours							
				Hepatocellular carcinomas		Cholangiocarcinomas		No. of animals with metastases			
		♂	♀	♂	♀	♂	♀	♂	♀	♂	♀
1	0 (control)	10	10	0	0	0	0	0	0	0	0
2	3,900	10	10	10	10	1	3	0	0	0	0
3	7,800	10	10	10	10	6	5	0	0	0	0
4	15,600	10	10	10	10	8	8	9	3	—	—
5	31,250	10	10	10	10	10	7	10	8	—	—
6	62,500	10	10	—	—	—	—	—	—	—	—

N-nitrosodiethanolamine was prepared on a large scale as follows. To a 6,000-ml Erlenmeyer flask cooled in an ice bath were added 1 kg diethanolamine (Aldrich), 1.24 kg sodium nitrite (Mallinckrodt) and 1.3 l water. After preliminary agitation to disperse the undissolved solid, the mixture was stirred magnetically overnight. To the homogeneous solution cooled to 0–5 °C in an ice bath was added 1.7 l 12 M HCl dropwise over a period of 4–5 h. Large volumes of brown nitrogen dioxide were evolved. On completion of the acid addition, stirring was continued for 1 h, after which 1,000 ml of methanol was slowly added to the still cold solution. Evolution of methyl nitrite ensued and dictated the rate of methanol addition. The flask was left overnight to complete transesterification. Salts present were removed by filtration using a fritted glass funnel. The filtrate was treated with small portions of a saturated sodium carbonate solution until a pH of 8 was attained. The colour of the solution changed from pale green to golden yellow. This solution was reduced to a viscous suspension by removing a large amount of water on a rotary evaporator. Anhydrous acetone was added to the suspension to decrease the viscosity and the salts in the suspension were removed by vacuum filtration. The filtrate was again reduced to a viscous suspension by removing acetone and residual water. To this suspension was added an equivalent volume of anhydrous acetone and the resulting mixture was filtered to remove the precipitated salts. Acetone was removed from the filtrate to give a golden yellow liquid of high viscosity. Analysis of this material by NMR spectrometry, IR spectrometry and UV spectrometry gave results consistent with the structure of nitrosodiethanolamine and indicated high purity. When 100 g was extracted four times with 100 ml of methylene chloride, the extract concentrated and the residue analysed by gas-liquid chromatography, no identifiable volatile nitrosamines were found at a detection level of 0.03 p.p.m.

each sex) 6 groups of 10. One of the groups served as untreated controls and the remainder were given solutions of nitrosodiethanolamine in neutral (pH 7) tap water that had been filtered through a deionizer. The concentrations of nitrosodiethanolamine and the numbers of animals treated are shown in Table 1.

Treatment consisted of giving the nitrosodiethanolamine solutions at 20 ml per rat each day on 5 days of each week; on the remaining 2 days the animals were given tap water without the nitrosamine. In these conditions almost all the nitrosamine solution was consumed, with little spillage (the tap water was given to replenish any water deficit suffered by the animals). Due to an error in communication, for the first 12 weeks of the experiment the nitrosamine solutions were supplied *ad libitum* for 7 days of each week; this hampered calculation of the exact doses received by each cage of animals, as the animals given the higher concentrations tended to drink more of the drug-containing solution (perhaps it has an appetizing taste). An exception was the 62,500 p.p.m. solution, of which little was drunk and which proved quite toxic, leading to the death of several animals within 2 weeks; treatment with this concentration of nitrosodiethanolamine was discontinued and the animals were discarded.

After 12 weeks the designated treatment regimen was restored and treatment was continued until 34 weeks after the beginning of the experiment. A few animals at the 31,250 and 15,600 p.p.m. concentrations died before the 34th week. All surviving animals were killed at week 34. The dead animals were completely necropsied and all lesions, with most normal tissues, were fixed for histological evaluation.

All the animals in the four long-term treated groups developed hepatocellular carcinomas of the liver. At the higher doses most rats also had cholangiocellular carcinomas. Histologically the hepatocellular carcinomas varied from well differentiated to poorly differentiated to undifferentiated, whereas the cholangiocarcinomas were well differentiated; each liver contained multiple large, invasive carcinomas. None of the untreated control animals had lesions of the liver. At the 31,250 and 15,600 p.p.m. doses, many of the liver carcinomas metastasized to the lungs and peritoneal cavity (Table 1).

Nitrosodiethanolamine is therefore a more potent carcinogen for the liver of Fischer 344 rats than was previously suspected. As liver carcinomas were induced after administration of a

solution containing 3,900 p.p.m. for only 34 weeks, it is likely that considerably lower concentrations will be effective. During most of the experiment the dose of nitrosodiethanolamine received by the animals was kept at 100 ml of solution per week, corresponding to 390 mg of nitrosodiethanolamine per week. This amounted to ~13 g per rat over the course of the treatment or 30 g per kg body weight for the males and 50 g per body weight for the females. This contrasts with the experiment of Druckrey *et al.*⁵, in which a total dose of 150–300 g of nitrosodiethanolamine per kg body weight gave rise to liver tumours in BD rats with a median induction time of 42 weeks.

Concentrations of nitrosodiethanolamine in some cutting oils reaches 3% or 30,000 p.p.m. (ref. 1) and in some cosmetics 48 p.p.m. (ref. 3). Therefore, the data presented here imply that the doses of this compound to which much of the human population is exposed can no longer be dismissed on the grounds that it is a weak carcinogen. Notably nitrosodiethanolamine has been found to penetrate rat skin quite readily⁸, so that skin contact with materials containing nitrosodiethanolamine is likely to present a carcinogenic risk to humans.

A similar study to that in rats was conducted with B6C3F₁ mice, groups of 10 female and 10 male mice being given nitrosodiethanolamine solutions in concentrations of 62,500–3,900 p.p.m. in water. In this case each mouse was allotted 5 ml of solution per day, 5 days a week for 32 weeks. Unlike the rats, mice tolerated the 62,500 p.p.m. concentration quite well. At the end of the study cholangiofibrosis and cirrhosis, as well as some hyperplastic nodules, were found in the livers of many mice receiving the higher doses of nitrosodiethanolamine, but no tumours were observed. This suggests that B6C3F₁ mice are less susceptible to the carcinogenic action of nitrosodiethanolamine than are F 344 rats.

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1. Fan, T. Y. *et al. Science* **196**, 70 (1977).
2. Zingmark, P. A. & Rappe, C. *Ambio* **6**, 237 (1977).
3. Fan, T. Y. *et al. Fd Cosmet. Toxicol.* **15**, 423 (1977).
4. Lijinsky, W., Keefer, L., Conrad, E. & Van de Bogart, R. *J. natn. Cancer Inst.* **49**, 1239 (1972).
5. Druckrey, H., Preussmann, R., Ivankovic, S. & Schmähel, D. *Z. Krebsforsch.* **69**, 103 (1967).
6. Hilfrich, J., Schmeltz, I. & Hoffmann, D. *Cancer Lett.* **4**, 55 (1978).
7. Rao, T. K., Young, J. A., Lijinsky, W. & Epler, J. L. *Mutat. Res.* **66**, 1 (1979).
8. Lijinsky, W., Losikoff, A. M. & Sansone, E. B. *J. natn. Cancer Inst.* (in the press).

Thyroid hormone modulation of X ray-induced *in vitro* neoplastic transformation

Duane L. Guernsey

Department of Biochemistry, College of Physicians and Surgeons, Columbia University, New York, New York 10032

Augustinus Ong & Carmia Borek

Radiological Research Laboratory, Department of Radiology and Department of Pathology, College of Physicians and Surgeons, Columbia University, New York, New York 10032

The direct oncogenic potential of X rays has been demonstrated by the *in vitro* neoplastic transformation of mammalian cells in culture¹, a technique which permits the study of oncogenesis in the absence of host-specific effects. Although several agents are known to modulate *in vitro* neoplastic transformation by X rays², little is known of the effects of hormones. We now describe experiments which show that the presence of thyroid hormone is necessary for *in vitro* neoplastic transformation by X rays in cells of an established mouse fibroblast culture (C3H/10T1/2) and in early-passage diploid hamster embryo cells.

Animal studies have shown that altered thyroid status modifies the growth and metastatic potential of implanted tumours⁵ as well as the survival rate of animals bearing tumours⁶. We therefore set out to investigate whether this modulation is a direct effect of the hormone on neoplastic transformation. *In vitro* transformation by X rays has been especially well investigated in early-passage primary hamster embryo cells in culture^{7,8} and C3H/10T1/2 mouse embryo fibroblasts⁹⁻¹¹, and qualitatively demonstrated in human diploid cells¹². Accordingly we have used in this study (1) short-term cultures of diploid hamster embryo cells (HE) obtained as previously described^{1,2,7,8}, and (2) the C3H/10T1/2 clone 8 mouse embryo heterodiploid cell line developed by Reznikoff *et al.*¹³.

We have measured the efficacy of X rays in inducing neoplastic transformations in cell cultures of both types when thyroid hormone has been removed from the culture medium and find

that transformation is inhibited. We have also shown that the efficacy of X rays in causing neoplastic transformation is restored when thyroid hormone (triiodothyronine, T₃) is added to the hormone-depleted medium.

Both cell types are fibroblast-like cells. Cell transformation was detected morphologically and scored as described elsewhere for the hamster embryo cells^{1,2,7,8} and for the C3H/10T1/2 mouse cells^{9-11,14}. In these earlier studies, the morphological changes associated with the transformed state have been correlated with the oncogenic potential of the cells. The procedures used are described in detail in Table 1 legend. Thyroid hormones (T₃ and thyroxine, T₄) were removed from heat-inactivated fetal bovine serum as described by Samuels *et al.*¹⁵ using AG1-X10 resin (Biorad). In each experiment, six different experimental culture conditions were used: (1) resin-treated serum (-T₃), unirradiated; (2) resin-treated serum (-T₃), irradiated; (3) resin-treated serum, T₃ (Sigma) added (+T₃), unirradiated; (4) resin-treated serum, T₃ added (+T₃), irradiated; (5) untreated serum, unirradiated; and (6) untreated serum, irradiated. Table 1 shows that when cells are maintained in media depleted of thyroid hormones (-T₃), X-ray irradiation-induced transformation is dramatically inhibited in both cell types tested. To support further the role of thyroid hormone in transformation, only T₃ was re-added to resin-treated, serum-supplemented media; the observed transformation frequency on re-addition was similar to control, irradiated cells maintained in untreated-serum-supplemented media.

A dose-response curve for cell survival was carried out, as described elsewhere^{7,8}, on each cell type to determine if the elimination of thyroid hormone would differentially affect the survival of the cells after X-ray irradiation. We found that the removal of thyroid hormones from the serum, or the resin treatment, had very little effect on the survival of either cell type (data not shown). Further, it was determined in both cell types that the growth characteristics of cells grown in the absence of T₃ were identical to the growth in the presence of T₃ in resin-treated, serum-supplemented media.

These data indicate that X-ray irradiation-induced *in vitro* transformation of mammalian cells is dramatically inhibited when cells are maintained in the absence of thyroid hormone. This seems to be a direct effect of the hormone as the expected frequency of transformation is restored when T₃ is reintroduced in the culture medium. These results cannot be ascribed to differences in survival or growth characteristics of the cells maintained in the presence or absence of thyroid hormone.

Table 1 The effect of thyroid hormone on X ray irradiation-induced cell transformation *in vitro* of C3H/10T1/2 mouse cells and hamster embryo cells in culture

Cells	Serum treatment/(T ₃ condition)	X rays (Gy)	Total surviving colonies	No. of colonies transformed	Transformation frequency
C3H/10T1/2	Untreated FBS	0	23,313	0	0
	Untreated FBS	3	27,737	24	8.65 × 10 ⁻⁴
	Resin-treated FBS/(-T ₃ -T ₄)	0	23,154	0	0
	Resin-treated FBS/(-T ₃ -T ₄)	3	33,747	0	0
	Resin-treated FBS + T ₃ /(+T ₃)	0	15,884	0	0
	Resin-treated FBS + T ₃ /(+T ₃)	3	21,040	16	7.60 × 10 ⁻⁴
HE	Untreated FBS	0	2,800	0	0
	Untreated FBS	2.2	4,800	16	3.33 × 10 ⁻³
	Resin-treated FBS/(-T ₃ -T ₄)	0	2,400	0	0
	Resin-treated FBS/(-T ₃ -T ₄)	2.2	1,500	0	0
	Resin-treated FBS + T ₃ /(+T ₃)	0	2,600	0	0
	Resin-treated FBS + T ₃ /(+T ₃)	2.2	3,600	15	4.17 × 10 ⁻³

Values given are totals of three separate experiments with C3H/10T1/2 cells and two separate experiments with hamster embryo cells. Stock 10⁻³ M T₃ in 50% *n*-propanol was diluted in media with 10% resin-treated fetal bovine serum (FBS) to give a final concentration of 10⁻⁷ M T₃ (designated +T₃). Media without thyroid hormone was prepared with 10% resin-treated FBS and an amount of diluent equal to that added in the +T₃ media. Stock cultures of C3H/10T1/2 cells were maintained in Eagle's basal medium +10% heat-inactivated FBS; hamster embryo cells in Dulbecco's modified Eagle's medium +10% FBS. Both cultures contained penicillin (50 U ml⁻¹) and streptomycin (50 µg ml⁻¹). All cells were maintained at 37 °C with 5% CO₂ in air throughout the experiment. One week before seeding, stock cultures were placed in the experimental culture media, with and without T₃ (as described in the text) and maintained in these conditions for the duration of the experiment. Twenty-four hours after seeding, the cells were irradiated with X rays at room temperature (2.2 Gy for HE and 3 Gy for C3H/10T1/2 cells), at a dose rate of 0.322 Gy min⁻¹, thereafter receiving weekly media changes. After an appropriate incubation period (6 weeks for C3H/10T1/2 and 2 weeks for HE), the cells were fixed and stained with Giemsa and scored for transformation. Both type II and III foci were scored in C3H/10T1/2 experiments.

Experiments, to be published in more detail elsewhere, indicate that when cells are exposed to concentrations of T_3 ranging from 10^{-11} M to 10^{-7} M, X-ray-induced transformation is dose dependent. Additionally, these studies demonstrate that the critical period of exposure to the hormone is a 12-h period before irradiation. This suggests that, in these experimental conditions, thyroid hormone is necessary for the induction and/or potentiation of X-ray irradiation-induced transformation *in vitro*. Thyroid hormone may also have a significant, perhaps even decisive, role in the occurrence of neoplastic transformation *in vivo*. Studies are in progress to define possible mechanisms of action and the involvement of thyroid hormone in neoplastic transformation *in vitro* by chemicals and viruses.

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1. Borek, C. & Sachs, L. *Nature* **210**, 276–278 (1966); *Proc. natn. Acad. Sci. U.S.A.* **57**, 1522–1527 (1967).
2. Borek, C. *Radiat. Res.* **79**, 209–232 (1979).
3. Dubnik, C. S., Morris, H. P. & Dalton, A. J. *J. natn. Cancer Inst.* **10**, 815–839 (1949).
4. Jabara, A. G. & Maritz, J. S. *Br. J. Cancer* **28**, 161–172 (1973).
5. Kumar, M. S., Chiang, T. & Deodhar, S. D. *Cancer Res.* **39**, 3515–3518 (1979).
6. Mishkin, S., Morris, H. P., Yalovsky, M. & Narisimha Murthy, P. V. *Cancer Res.* **39**, 2371–2375 (1979).
7. Borek, C. & Hall, E. J. *Nature* **243**, 450–453 (1973); *Nature* **252**, 499–501 (1974).
8. Borek, C., Hall, E. J. & Rossi, H. H. *Cancer Res.* **38**, 2997–3005 (1978).
9. Terzaghi, M. & Little, J. B. *Cancer Res.* **36**, 1367–1374 (1976).
10. Han, A. & Elkind, M. M. *Cancer Res.* **39**, 123–130 (1979).
11. Borek, C., Miller, P., Pain, C. & Troll, W. *Proc. natn. Acad. Sci. U.S.A.* **76**, 1800–1803 (1979).
12. Borek, C. *Nature* **283**, 776–778 (1980).
13. Reznikoff, C. A., Bronkow, D. W. & Heidelberger, C. *Cancer Res.* **33**, 3231–3238 (1973).
14. Reznikoff, C. A., Bertram, J. S., Bronkow, D. W. & Heidelberger, C. *Cancer Res.* **33**, 3239–3249 (1973).
15. Samuels, H. H., Stanley, F. & Casanova, J. *Endocrinology* **105**, 80–85 (1979).

Friend leukaemia virus-transformed cells, unlike normal stem cells, form spleen colonies in Sl/Sl^d mice

Dixie Mager, Tak W. Mak & Alan Bernstein

The Ontario Cancer Institute and Department of Medical Biophysics, University of Toronto, Toronto, Ontario, Canada M4X 1K9

Neoplastic cells are characterized by partial or total autonomy from the interactions that regulate the behaviour of normal cells in the intact animal. Despite the role of the host cellular environment in governing the proliferation and differentiation of both normal and malignant cells, little is actually known about these host factors. The characterization of host genes that influence both normal cellular processes, as well as susceptibility to tumour induction, is one approach to identifying such factors. Mice carrying two recessive mutations at the *steel* (*Sl*) locus have an environmental defect that affects both normal haematopoietic stem cell function^{1,2} as well as susceptibility to Friend leukaemia virus^{3–5}. In this study, we have used Sl/Sl^d mice to examine whether malignant transformation by this RNA tumour virus results in a population of cells capable of proliferating even in the defective cellular microenvironment of Sl/Sl^d mice. We report here that late after infection, the leukaemic spleens of Friend virus-infected mice contain cells which, unlike normal haematopoietic stem cells, are able to form macroscopic spleen colonies in irradiated mice of genotype Sl/Sl^d . This observation forms the basis for the first *in vivo* colony assay for leukaemic cells transformed by Friend virus.

Mice carrying two mutant alleles at the *steel* locus (Sl/Sl^d) exhibit pleiotropic defects, including macrocytic anaemia, sterility and defective pigmentation⁶. Transplantation studies have suggested that the haematopoietic defect in these mice is in the cellular microenvironment in which normal haematopoietic stem cells, and more committed erythroid progenitors, proliferate and differentiate. Haematopoietic cell populations from Sl/Sl^d mice contain normal numbers of stem cells, as measured by their ability to form spleen colonies (colony-forming unit-spleen, CFU-S⁷) in irradiated $+/+$ littermates. Thus, the *Sl* locus seems to specify a function, presumably involving short-range cell-cell contacts^{8,9}, that regulates the ability of pluripotent stem cells to undergo normal erythropoietic differentiation.

Genetically anaemic Sl/Sl^d mice are also highly resistant to spleen focus induction by the polycythaemia- and anaemia-inducing isolates of Friend spleen focus-forming virus^{3–5}. The basis for this resistance is unknown. We presented evidence previously that the spleens from $+/+$ mice, infected up to 2 weeks previously with Friend virus, did not contain cells capable of spleen colony formation in irradiated Sl/Sl^d mice⁴. Friend leukaemia seems to be a multi-stage disease in which truly malignant cells capable of extensive proliferation do not appear until late in the disease^{10,11}. Thus, the failure to observe spleen colonies in secondary Sl/Sl^d recipients early after infection of $+/+$ mice could be attributed to either the absence of any leukaemic stem cells in the $+/+$ donor spleens or the inability of Friend virus-transformed cells to proliferate in the *steel* environment.

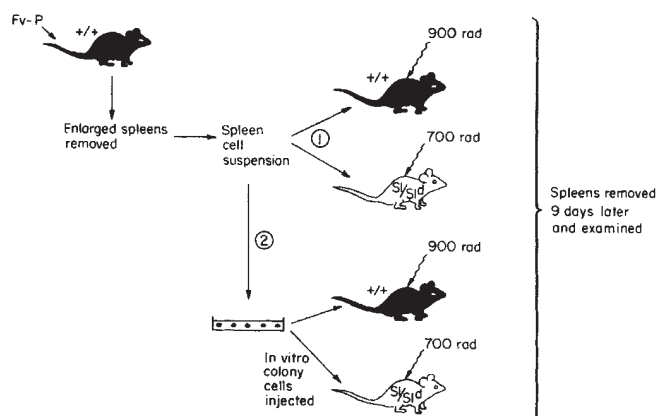


Fig. 1 Experimental protocols used to study spleen colony forming ability of Friend virus-infected $+/+$ spleen cells. Normal WCB6 F₁ mice of $+/+$ genotype (obtained from crossing mice of strains WC/Re-S1/+ and C57BL/6J- $+/Sl^d$) were injected intravenously (i.v.) with 10^3 focus-forming units (FFU) of SFFV_P (spleen focus-forming virus contained in preparations of FV-P). SFFV_P titres were determined in DBA/2J mice by the spleen-focus assay method²². At various times after infection, spleens were removed and single-cell suspensions made in Iscove's Modified Dulbecco's Medium (IMDM) and 5% heat-inactivated fetal calf serum. In the first procedure, the spleen cells were injected i.v. into lethally irradiated $+/+$ or Sl/Sl^d recipients. (Mice were irradiated immediately before injection using a ¹³⁷Cs biological irradiator.) Nine days later the spleens of recipient mice were fixed in Bouin's solution and examined for spleen colonies. In the second procedure, the spleen cells were plated in methylcellulose using a procedure described elsewhere¹⁵. In brief, 10^6 viable nucleated cells per ml were plated in 2.0% methylcellulose in IMDM (containing 2×10^{-5} M 2-mercaptoethanol) plus 30% heat-inactivated fetal calf serum. Large spherical colonies (containing 2×10^4 – 1×10^5 cells) were picked 14 days later. Colonies were pooled and equal numbers of viable nucleated cells were injected i.v. into irradiated $+/+$ or Sl/Sl^d mice. Spleens were examined 9 days later.

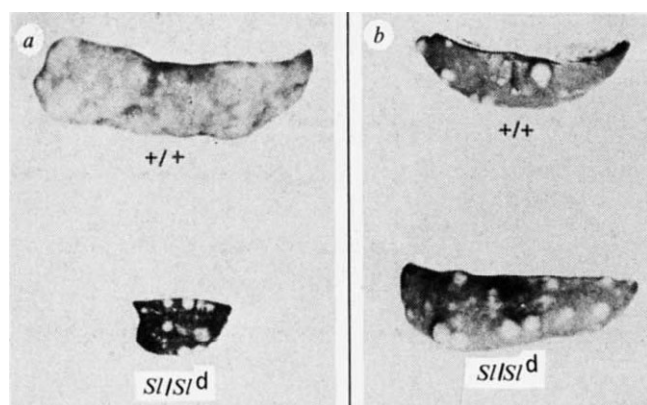


Fig. 2 Appearance of spleen colonies formed by Friend leukaemia virus-transformed $+/+$ spleen cells obtained from *a*, primary leukaemic spleens or *b*, colonies in methyl cellulose derived from a leukaemic spleen. *a*, Viable nucleated spleen cells (5×10^6) from a $+/+$ mouse infected 10 weeks previously with FV-P were injected into irradiated $+/+$ or SI/SI^d recipients. Spleens were fixed in Bouin's solution 9 days later. The upper spleen, from a $+/+$ recipient, is confluent with CFU-S colonies. The lower spleen (approximately half of total) is from an SI/SI^d recipient. Sixteen spleen colonies were counted on this spleen section. *b*, Viable nucleated cells (2.5×10^5) from the pooled *in vitro* colonies, described in Fig. 1 legend, derived from a $+/+$ mouse infected 9 weeks previously with FV-P, were injected into irradiated $+/+$ or SI/SI^d recipients. Spleen colonies were counted 9 days later. 25 colonies were counted on the $+/+$ recipient spleen and 31 colonies were found on the SI/SI^d recipient spleen.

To examine the influence of the *steel* environment on both the early and late stages of Friend leukaemia, the ability of $+/+$ spleen cells from Friend virus-infected mice to proliferate and form macroscopic spleen colonies in irradiated SI/SI^d was measured. Two experimental approaches, outlined in Fig. 1, were followed. In the first procedure, spleen cells, derived from the enlarged spleens of $+/+$ mice infected at various times previously with a clonal isolate¹² of the polycythaemia-inducing strain of Friend leukaemia virus (FV-P), were injected into either irradiated $+/+$ or SI/SI^d secondary recipients. As shown in Table 1, spleen cells from either normal $+/+$ mice or $+/+$ mice infected up to 5 weeks earlier with FV-P did not contain cells capable of spleen colony formation in irradiated SI/SI^d mice, whereas these same cell populations did contain CFU-S capable of forming spleen colonies in irradiated $+/+$ littermates. These observations agree with previous findings that CFU-S cannot form spleen colonies in SI/SI^d mice¹, and extend previous observations from this laboratory that, early after infection with FV-P, the spleens of $+/+$ mice do not contain cells capable of forming spleen colonies in SI/SI^d mice⁴.

Table 1 Spleen colony formation by Friend leukaemia virus-transformed spleen cells

Time after infection	No. of spleen cells injected per mouse	No. of spleen colonies $+/+$ recipients	No. of spleen colonies SI/SI^d recipients
Uninfected	2×10^6	TMTC* (3)	0 (3)
10 days	2×10^6	TMTC (3)	0 (12)
5 weeks	5×10^6	TMTC (3)	0 (3)
10 weeks	5×10^6	TMTC (3)	24 ± 8 (3)

Viable nucleated cells from spleens of uninfected $+/+$ mice or mice infected with FV-P for various times were injected intravenously into lethally irradiated $+/+$ or SI/SI^d recipients. Spleens were removed 9 days later. The number of recipient spleens examined in each group is enclosed in parentheses. In every case, the spleens of $+/+$ recipients were confluent with CFU-S colonies.

* Too many to count.

However, at a later time after infection (10 weeks), the $+/+$ leukaemic spleens did contain cells that were now able to form macroscopic spleen colonies in irradiated SI/SI^d hosts (Fig. 2*a* and Table 1). Macroscopically, the nodules or colonies formed by $+/+$ leukaemic spleen cells in irradiated SI/SI^d mice were less diffuse than, and could be easily distinguished from, both smaller FV-P induced spleen foci, and larger CFU-S derived colonies (Fig. 2*a*). In addition, as shown here and previously^{1,4}, CFU-S does not form spleen colonies in SI/SI^d hosts, nor can FV-P-infected cells act as infectious centres in an irradiated host. Thus, the colonies observed in the irradiated SI/SI^d mice are derived from a new cell type that only appears very late after infection with FV-P. One such colony, when placed in cell culture, gave rise directly to a permanent cell line. The properties of this cell line are very similar to cell lines derived from leukaemic spleens after subcutaneous injections into syngeneic secondary recipients^{13,14}—the cells grow in suspension, resemble immature erythroid cells, and can be induced to synthesize haemoglobin after treatment with dimethyl sulphoxide (data not shown). Thus, spleen colony formation in irradiated SI/SI^d mice by $+/+$ leukaemic spleens provides an *in vivo* assay system, free of both an infectious centre and CFU-S background, for the enumeration of a new colony-forming cell that results from transformation by Friend leukaemia virus.

These experiments clearly indicate that, late after infection, Friend virus-infected leukaemic spleens contain cells that can proliferate in the defective SI/SI^d environment. However, the relative colony-forming efficiency of these cells in $+/+$ and SI/SI^d mice is unknown, because of the CFU-S background when $+/+$ mice are used as hosts (Table 1). Therefore, the second experimental protocol outlined in Fig. 1 legend was followed. In this procedure, spleen cells from $+/+$ mice infected 9 weeks previously with FV-P were first cloned in a semi-solid methylcellulose medium in the absence of any exogenous conditioning factors. This *in vitro* assay, which is described more fully elsewhere¹⁵, seems to detect a population of colony-forming cells similar to that detected by the *in vivo* SI/SI^d assay described above. In brief, these *in vitro* colony-forming cells can only be detected in the advanced stages of Friend disease (greater than 3 weeks) and not in uninfected spleens or in spleens early after infection with Friend virus. The cells in the *in vitro* colonies form spleen colonies in irradiated syngeneic hosts, are tumorigenic and a significant proportion can give rise directly to permanent erythroid cell lines¹⁵. These cell lines can also be chemically induced to synthesize haemoglobin.

As outlined in Fig. 1 legend, *in vitro* colonies derived from the leukaemic spleens of FV-P-infected $+/+$ mice were pooled and equal numbers of cells injected into either irradiated $+/+$ or SI/SI^d hosts. As shown in Fig. 2*b*, approximately equal numbers of macroscopic spleen colonies were observed 9 days later in both normal and genetically anaemic littermates. Furthermore, both the size and macroscopic appearance of the spleen colonies were identical in both sets of mice.

To obtain more quantitative data on the relative colony-forming ability of these FV-P-transformed $+/+$ cells in $+/+$ and SI/SI^d hosts, varying numbers of cells, from a cell line that had been established in suspension culture for 6 weeks from a primary colony in methylcellulose, were injected into lethally irradiated $+/+$ or SI/SI^d recipients. As shown in Fig. 3, the spleen colony-forming ability in either host was a linear function of the number of cells injected, with similar slopes in $+/+$ and SI/SI^d recipients.

The apparent multi-stage nature of the erythroleukaemia induced by Friend virus has been reported^{10,11}. The early stage of Friend disease is characterized by a rapid increase in the number of erythroid colony-forming cells^{16,17} with limited proliferative capacity, whereas the late stage is associated with the appearance of spleen cells capable of forming solid tumours in secondary recipients or permanent cell lines in culture^{11,13,18,19}. Investigation of the cellular relationship between these stages has been difficult due to the lack of a clonal and quantitative assay for these Friend virus-transformed tumour cells. The

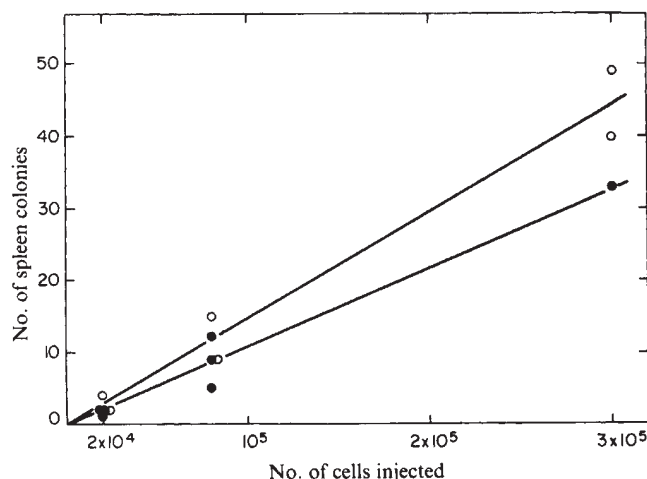


Fig. 3 Spleen colony formation by a Friend leukaemia virus-transformed cell line in irradiated $+/+$ and SI/SI^d recipients. The cell line was derived 6 weeks previously from an *in vitro* colony obtained by plating spleen cells from a $+/+$ mouse infected 10 weeks previously with FV-P. Various numbers of cells were injected i.v. into irradiated $+/+$ (●) or SI/SI^d (○) recipients. Nine days later, spleens were fixed in Bouin's solution and spleen colonies counted.

results presented here indicate that irradiated SI/SI^d mice are suitable hosts for the definitive enumeration of a class of cells with extensive proliferative capacity that only appear quite late after infection with FV-P. The use of SI/SI^d secondary recipients eliminates any background colonies contributed by CFU-S in the donor spleens. In addition, although virus is released from the $+/+$ donor spleen cells, use of irradiated secondary recipients eliminates the background of virus-infected spleen foci of host origin⁴ which can arise in unirradiated recipients, even those restrictive for viral replication^{20,21}. Thus, this quantitative colony assay should facilitate analysis of the relationship between the early and late stages of Friend leukaemia.

That Friend virus-transformed $+/+$ cells can form spleen colonies with equal efficiency in both $+/+$ and SI/SI^d hosts suggests that these erythroleukaemic cells, unlike normal erythroid progenitor cells, are autonomous of the environmental defect in SI/SI^d mice. However, mutant SI/SI^d mice are highly resistant to spleen focus and polycythaemia induction by FV-P, indicating that mutation at the *steel* locus can affect at least the early stages of Friend disease. Thus, the observation that Friend virus-transformed $+/+$ spleen cells can proliferate to form spleen colonies in SI/SI^d mice raises the intriguing possibility that progression from the early to late stages of Friend leukaemia is accompanied by the appearance of cells with not only extensive proliferative capacity but also autonomy from a genetically defined cellular regulatory factor.

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- McCulloch, E. A., Siminovitch, L., Till, J. E., Russell, E. S. & Bernstein, S. E. *Blood* **26**, 399–410 (1965).
- Russell, E. S. *Adv. Genet.* **20**, 357–459 (1979).
- Bennett, M., Steeves, R. A., Cudkovic, G., Mirand, E. A. & Russell, L. B. *Science* **162**, 564–565 (1968).
- McCool, D., Mak, T. W. & Bernstein, A. *J. exp. Med.* **149**, 837–846 (1979).
- MacDonald, M. E. *et al. J. exp. Med.* **151**, 1477–1492 (1980).
- Russell, E. S. & Bernstein, S. E. in *The Biology of the Laboratory Mouse* (ed. Green, E. L.) 351–372 (McGraw-Hill, New York, 1966).
- Till, J. E. & McCulloch, E. A. *Radiat. Res.* **14**, 213–222 (1961).
- Bernstein, S. E. *Am. J. Surg.* **119**, 448–451 (1970).
- Trentin, J. J. *et al. in Morphological and Functional Aspects of Immunity* (eds Landahl-Kiessling, K. *et al.*) 289–298 (Plenum, New York, 1971).
- Tambourin, P. E., Wendling, F., Jasmin, C. & Smadja-Joffe, F. *Leukemia Res.* **3**, 117–129 (1979).

- Levy, S. B., Blankstein, L. A., Vinton, E. C. & Chambers, T. J. in *Oncogenic Viruses and Host Cell Genes* (eds Ikawa, Y. & Odaka, T.) 409–428 (Academic, New York, 1979).
- Bernstein, A., Mak, T. W. & Stephenson, J. R. *Cell* **12**, 287–294 (1977).
- Friend, C. & Haddad, J. R. *J. natn. Cancer Inst.* **25**, 1279–1289 (1960).
- Friend, C., Scher, W., Holland, J. C. & Sato, T. *Proc. natn. Acad. Sci. U.S.A.* **68**, 378–382 (1971).
- Mager, D., Mak, T. W. & Bernstein, A. *Proc. natn. Acad. Sci. U.S.A.* (in the press).
- Liao, S. K. & Axelrad, A. A. *Int. J. Cancer* **15**, 467–482 (1975).
- Horoszewicz, J. S., Leong, S. S. & Carter, W. A. *J. natn. Cancer Inst.* **54**, 265–267 (1975).
- Ostertag, W., Melderis, H., Steinheider, G., Kluge, N. & Dube, S. *Nature new Biol.* **239**, 231–234 (1972).
- Tambourin, P. *et al. in In vivo and in vitro Erythropoiesis: The Friend System* (ed. Rossi, G. B.) 127–138 (Elsevier, Amsterdam, 1980).
- Steeves, R. A., Bubbers, J. E., Plata, F. & Lilly, G. *Cancer Res.* **38**, 2729–2733 (1978).
- Wendling, F. & Tambourin, P. E. *Int. J. Cancer* **22**, 479–486 (1978).
- Axelrad, A. A. & Steeves, R. A. *Virology* **24**, 513–518 (1964).

Interferon suppresses antigen- and mitogen-induced leukocyte migration inhibition

R. Szigeti, Maria G. Masucci, G. Masucci, Eva Klein & G. Klein

Department of Tumor Biology, Karolinska Institute, S-104 01 Stockholm 60, Sweden

W. Berthold

Department of Biological Research, D-7950 Biberach/Riss, FRG

Although first recognized by its effect on virus-cell interactions, interferon (IFN)¹ has a variety of other effects². It can affect cell proliferation^{3,4}, modify the immune response at several levels⁵, enhance the cytotoxic action of lymphocytes^{6–9}, suppress antibody formation¹⁰ and inhibit the development of delayed-type hypersensitivity (DTH) reactions^{11,12}. Therefore we have now tested the effect of interferon on leukocyte migration inhibition (LMI), regarded as the counterpart *in vitro* of DTH in humans¹³. We have found that IFN suppresses both mitogen- and antigen-induced LMI, acting directly on the granulocytes but also affecting the lymphokine production of the lymphocytes.

The agarose microdroplet technique¹⁴ was used in two systems: migration inhibition induced by phytohaemagglutinin (PHA) and LMI of leukocytes from Epstein-Barr virus (EBV)-seropositive donors after exposure to EBV antigens. Washed buffy coat cells (20×10^6) were mixed with $135 \mu\text{l}$ nutrient agarose medium containing an equal volume of $2 \times \text{RPMI } 1640$ medium, supplemented with 20% FCS or NCS and 0.4% agarose. Droplets ($2 \mu\text{l}$) were placed in migration chambers (Sterilin) and after they had solidified, the chambers were filled with medium, with or without mitogen/antigen and incubated in a 37°C humidified CO_2 atmosphere for 18–24 h. After incubation the migration areas were measured and the % migration inhibition (MI) calculated according to the formula

$$\% \text{ MI} = \left[1 - \frac{\text{mean migration area with antigen}}{\text{mean migration area without antigen}} \right] \times 100$$

Positive MI was recorded when the % inhibition exceeded 20 in the antigen-induced system and 50 with PHA. Purified PHA (Wellcome), $1 \mu\text{g ml}^{-1}$, is known to induce LMI of cells from healthy donors^{15,16}. We have previously shown that crude extracts of EBV genome-carrying cells ($50 \mu\text{g}$ per ml protein) or partially purified EBV-determined nuclear antigen (EBNA), $10 \mu\text{g ml}^{-1}$, inhibits the leukocyte migration in EBV-seropositive healthy individuals^{17,18}. In this study we used a crude extract of the EBV-producer P3HR-1 line and a partially purified¹⁹ EBNA preparation. Human leukocyte interferon (Hu-IFN- α), further IFN was derived from Namalwa cells induced by Sendai virus²⁰. The preparation contained $2.2 \times$

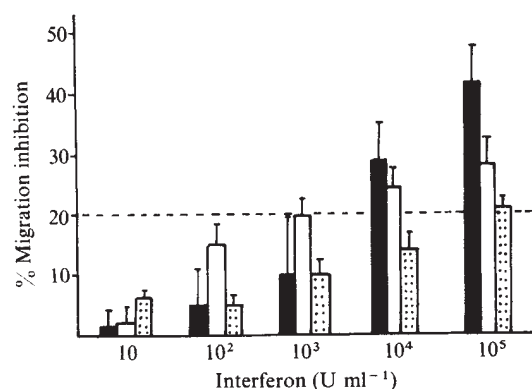


Fig. 1 Effect of IFN on the migration of buffy coat cells (■, 16 experiments) and separated granulocytes (□, 5 experiments). The results are expressed as mean $\pm$ s.e.m. migration inhibition; 20% inhibition (horizontal dotted line) can be regarded as significant. (▨) represents the mean percentage ($\pm$ s.e.m.) of dead cells among Ficoll-separated lymphocytes after 24 h cultivation in the presence of various concentrations of IFN.

10⁵ U per mg protein. The same batch of IFN was also effective in natural killer cell (NK) boosting experiments²¹.

Figure 1 shows that IFN inhibits leukocyte migration when used in high concentrations (10⁴–10⁵ U ml⁻¹), probably due to its cytotoxic effect on the lymphocytes, as detected by trypan blue exclusion tests in six experiments. At 100 U ml⁻¹, IFN had no significant effect on LMI or detectable cytotoxic effect—the viability of the lymphocytes was 95.8 $\pm$ 2.1% after 24 h cultivation.

PHA- and antigen-induced LMI was reduced in the presence of 100 U per ml IFN (Table 1). In the PHA system the mean % MI decreased to 32% compared with 61% in the absence of

Table 1 PHA- and EBV-antigen-induced LMI in the presence of 100 U per ml IFN

PHA (1 μ g ml ⁻¹)		P3HR-1 crude extract (50 μ g ml ⁻¹)		Partially purified EBNA (10 μ g ml ⁻¹)	
-IFN	+IFN	-IFN	+IFN	-IFN	+IFN
50	32	Seronegative healthy persons			
61	45	9	17	16	20
58	35	10	16	29	25
71	50	14	-7*	14	10
52	12	17	12	21	23
59	40				
66	48	12.5 $\pm$	9.5 $\pm$	20.0 $\pm$	19.5 $\pm$
55	37	2.13 [†]	2.59	3.81	3.89
60	6				
69	41	Seropositive healthy persons			
52	-2*	72	15	28	-1
66	52	50	18	58	25
73	32	45	14	49	9
50	11	39	-3	35	-5
66	42	48	-10	33	20
		46	-10	39	13
60 $\pm$	32.0 $\pm$	43	13	50	10
2.05	4.78	48	-6	45	-2
		40	7	59	7
		40	27	ND	ND
		42	21	42	8
		49	7	46	23
		46.8 $\pm$	7.75 $\pm$	44.0 $\pm$	8 $\pm$
		2.62	2.08	2.96	1.26

Values are % inhibition of migration. ND, not detected.

*-inhibition = stimulation of migration.

[†] Mean $\pm$ s.e.m.

IFN. Migration of leukocytes from EBV-seronegative persons was not influenced by EBV-antigen preparations nor by addition of 100 U per ml IFN. The leukocyte migration of EBV-seropositive individuals was inhibited by the two antigen preparations (47% and 44% LMI was induced by the crude P3HR-1 cell extract and by the partially purified EBNA preparation, respectively). Migration inhibition was almost completely abolished in the presence of IFN—this effect was also observed when Hu-IFN- α ¹ polypeptide²², derived from *Escherichia coli*, was tested in the LMI system²³.

The direct LMI system is complex: the buffy coat contains both the leukocyte migration inhibitory factor (LIF), producer lymphocytes and the indicator (migrating) granulocytes. To determine whether IFN acted on the lymphocyte and/or granulocyte component, we tested the effect of IFN on the migration of separated granulocytes. IFN at the standard dose used in the mitogen and antigen assays (100 U ml⁻¹) was slightly inhibitory (Fig. 1). This result suggests that the IFN-mediated decrease of LMI was not due to stimulation of granulocyte movement. In this system, as in other experiments¹⁶, 1 μ g per ml PHA had no direct effect on the granulocyte migration. However, addition of lymphokine-containing supernatant of PHA-stimulated lymphocyte cultures inhibited the migration of separated granulocytes (indirect LMI assay) (Fig. 2).

The supernatant-induced migration inhibition was abolished by adding 100 U per ml IFN during the migration period (mean % MI decreased to 18.3% compared with 42.5% in the absence of IFN) (Table 2). Furthermore, 30 min incubation with IFN before addition of the PHA supernatant had the same blocking effect. In the reversed situation (incubation with IFN 30 min after the addition of PHA supernatant) the supernatant-induced migration inhibition was not abolished. These data indicate that IFN blocks the effect of previously released LIF on the granulocytes. This can be due to competition between LIF and IFN for receptor structures present on the granulocyte membranes. Competition of this type was found to occur between IFN and PHA or other substances (such as cholera and tetanus toxin and certain hormones) when measuring the binding of IFN to cell membrane gangliosides (for review see ref. 24).

The indirect LMI assay (granulocyte migration in the presence of lymphokine-containing supernatants, produced by mitogen-stimulated lymphocytes) was also used to detect the effect of IFN on the lymphocyte component. Untreated and mitomycin-C treated lymphocytes were stimulated with PHA, in the presence and absence of 100 U per ml IFN. The IFN specificity of the effect was tested by adding anti-IFN serum

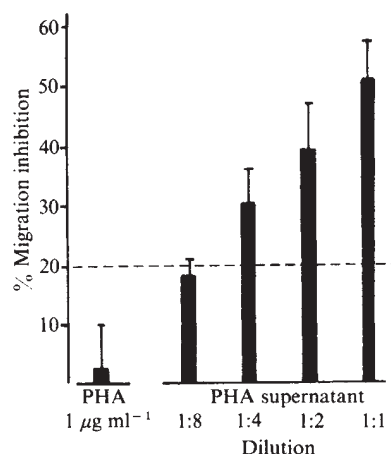


Fig. 2 Inhibitory effect of PHA and PHA supernatant on the migration of separated granulocytes (eight experiments). The results are expressed as mean $\pm$ s.e.m. % migration inhibition. 20% inhibition (horizontal dotted line) can be regarded as significant.

(provided by K. Cantell). Table 3 shows the results of five experiments. They show that non-proliferating lymphocytes can produce LIF, in analogy with the production of macrophage migration inhibitory factor (MIF)²⁵. This precludes the possibility that the IFN-mediated suppression of LMI could be due to its growth-inhibitory effect on the LIF-secreting lymphocytes. In addition, LIF production by both untreated and mitomycin-treated lymphocytes was suppressed by 100 U per ml IFN: 37.6% against 9.2% in the untreated and 31.6% against 8.6% LMI in mitomycin-treated lymphocytes (Table 3). The IFN-induced suppression of LIF production by untreated lymphocytes was completely prevented by the simultaneous addition of anti-IFN serum (37.6% against 9.2%), confirming that IFN itself was responsible for the suppression of LIF production.

Table 2 Effect of IFN on the granulocyte migration inhibition induced by lymphokine-containing PHA supernatant

Donor	-IFN	+IFN*	+ 30 min IFN pretreatment†	+ IFN 30 min post-treatment‡
1	47	24	ND	ND
2	52	5	ND	ND
3	39	6	18	35
4	35	13	13	28
5	43	26	19	39
6	37	9	19	42
7	43	29	23	41
8	49	21	25	52
9	36	21	20	38
10	44	17	24	38
	42.5 ± 1.9	18.3 ± 2.7	20.1 ± 1.5	39.1 ± 2.6

Values are % inhibition of migration, those at the base of the columns are mean ± s.e.m.

* Lymphokine production was induced in blood lymphocytes by PHA stimulation. 2×10^6 lymphocytes were resuspended in RPMI 1640 supplemented with 10% FCS. PHA ($1 \mu\text{g ml}^{-1}$) was added to the culture. The LIF-containing supernatant was collected after 36–48 h and tested for its ability to induce LMI on purified granulocytes as previously described. 100 U per ml of IFN were added during the migration period together with the PHA supernatant (column 2), 30 min before or after PHA supernatant addition (columns 3 and 4, respectively).

† Lymphocytes were preincubated with IFN for 30 min.

‡ IFN was added 30 min after start of culture.

Table 3 Effect of IFN and anti-IFN serum on LIF production by untreated and mitomycin-treated lymphocytes

Donor	Untreated cells			Mitomycin-treated* cells	
	PHA	PHA+ IFN†	PHA+ IFN + anti-IFN‡	PHA	PHA+ IFN
1	39	10	32	34	12
2	38	13	45	29	0
3	27	0	38	18	7
4	39	12	33	36	11
5	45	11	40	41	13
	37.6 ± 3.2	9.2 ± 2.63	37.6 ± 2.66	31.6 ± 4.37	8.6 ± 2.66

Values are % migration inhibition induced on separated granulocytes by lymphocyte culture supernatants, produced in various culture conditions. Values at the base of the columns are mean ± s.e.m.

* Lymphocytes were incubated with $25 \mu\text{g ml}^{-1}$ mitomycin-C (Sigma) for 30 min, then the cells were washed thoroughly with medium three times and cultivated for LIF production as described in Table 2.

† 100 U ml^{-1} IFN was added during the secretion period.

‡ Anti-(100 U ml^{-1}) IFN antiserum was added simultaneously. Addition of the same amount of antiserum to the migrating granulocytes had no toxic effect (MI was 10.5%, not significantly different from the control). The sheep anti-IFN serum has a titre of 4.5×10^5 antiviral units per ml.

Table 4 Effect of preincubation with IFN or IFN + anti-IFN serum on LIF production of lymphocytes after PHA stimulation

Donor	-IFN	+IFN	+IFN + anti-IFN
1	52	24	ND
2	41	15	44
3	41	10	36
4	42	18	32
	44 ± 3.09	16.75 ± 5.85	37.3 ± 4.3

The cells were incubated for 30 min with 100 U ml^{-1} IFN, with or without simultaneous addition of anti-(100 U ml^{-1}) IFN serum, then washed thoroughly with medium three times and cultivated with $1 \mu\text{g ml}^{-1}$ PHA-P (see Table 2 for culture conditions). Values are % migration inhibition induced on separated granulocytes, by lymphocyte culture supernatants produced in various culture conditions. Values at the base of the columns are mean ± s.e.m. ND, not detected.

In these experiments IFN was present during the migration period, thus a direct effect on the migrating granulocytes cannot be ruled out. Therefore, in the final experiments lymphocytes were preincubated for 30 min with IFN (with or without anti-IFN serum) and subsequently washed before the PHA-stimulation. The results (Table 4) show that preincubation with 100 U per ml IFN was sufficient to reduce significantly LIF production of PHA-activated lymphocytes (44% MI in the untreated and 16.7% in the IFN-pretreated lymphocytes). The suppression of LMI was prevented by the simultaneous addition of anti-IFN serum (37.3% against 16.7%).

These data suggest that IFN has a direct effect on the granulocytes in the LMI system and that it also blocks LIF production by the activated lymphocytes. This could be due to a direct inhibition of the cellular protein synthesis²⁶ or due to the activation of suppressor cells²⁷. We suggest that the latter mechanism could operate *in vivo*, for example in relation to the immunodepression observed during the acute phase of infectious mononucleosis^{16,28}. An IFN-like factor in the serum of acute mononucleosis patients was actually found to block LMI²⁹.

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- Interferon Nomenclature *Nature* **286**, 110 (1980).
- Gresser, I. *Cell Immun.* **34**, 406–411 (1977).
- Gresser, I., Brouty-Boye, D., Thomas, M. T. & Macieira-Coelho, A. *Proc. natn. Acad. Sci. U.S.A.* **66**, 1052–1058 (1970).
- Adams, A., Strander, H. & Cantell, K. *J. gen. Virol.* **28**, 207–217 (1975).
- Epstein, L. in *Interferons and their Actions* (ed. Stewart, W. E.) 91–132 (CRB Press, Ohio, 1977).
- Trinchieri, G. & Santoli, D. *J. exp. Med.* **147**, 1314–1333 (1978).
- Einhorn, S., Blomgren, H. & Strander, H. *Int. J. Cancer* **22**, 405–412 (1978).
- Gidlund, M., Örn, A., Wigzell, H., Senik, A. & Gresser, I. *Nature* **273**, 759–761 (1978).
- Herberman, R. B., Ortaldo, J. R. & Bonnard, G. *Nature* **277**, 221–223 (1979).
- Johnson, H. M., Smith, B. G. & Baron, S. *J. Immun.* **114**, 403–409 (1975).
- Hirsch, M. S., Ellis, D. A. & Black, P. H. *Transplantation* **17**, 234–236 (1974).
- De Maeyer, E., De Maeyer-Guignard, J. & Vandeputte, M. *Proc. natn. Acad. Sci. U.S.A.* **72**, 1753–1757 (1975).
- Søborg, M. & Bendixen, G. *Acta med. scand.* **181**, 247–256 (1967).
- McCoy, J. L., Dean, J. H. & Herberman, R. B. *J. immun. Meth.* **15**, 355–371 (1977).
- Morison, W. L. *J. clin. Path.* **27**, 113–117 (1974).
- Szigeti, R., Bokay, J., Revesz, T. & Schuler, D. *Allergy* (in the press).
- Szigeti, R., Timar, L. & Revesz, T. *Allergy* **35**, 97–103 (1980).
- Szigeti, R., Luka, J. & Klein, G. *Cell Immun.* (in the press).
- Luka, J., Lindahl, T. & Klein, G. *J. Virol.* **27**, 604–611 (1978).
- Bodo, G. in *Acad. Sci. Acts. Proc. Symp. Preparation, Standardization and Clinical Use of Interferon*, Zagreb (ed. Ikie, D.) 49–57 (1977).
- Masucci, M. G., Masucci, G., Klein, E. & Berthold, W. *Proc. natn. Acad. Sci. U.S.A.* **77**, 3620–3624 (1980).
- Nagata, S. *et al. Nature* **284**, 316–320 (1980).
- Masucci, M. G. *et al. Science* **209**, 1431–1435 (1980).
- Besancon, F. & Ankel, H. *Tex. Rep. Biol. Med.* **35**, 282–289 (1977).
- Rocklin, R. E. *J. Immun.* **110**, 674–678 (1973).
- Fuse, A. & Kuwata, T. *J. gen. Virol.* **33**, 17–24 (1976).
- Kadish, A. S., Tansey, F. A., Yu, G. S. M., Doyle, A. T. & Bloom, B. R. *J. exp. Med.* **151**, 637–650 (1980).
- Mangi, R. J. *et al. New Engl. J. Med.* **291**, 1149–1153 (1974).
- Lai, P. K., Alpers, M. P. & Mackay-Scolay, E. M. *Int. J. Cancer* **20**, 21–29 (1977).

Prostaglandin modulation of development of cell-mediated immunity in culture

Kam H. Leung & Enrico Mihich

Department of Experimental Therapeutics, Grace Cancer Drug Center, Roswell Park Memorial Institute, New York State Department of Health, Buffalo, New York 14263

Prostaglandins (PGs) have been implicated as possible modulators of the immune response and various inflammatory processes¹⁻⁴. Various cell components of the immune system are sources of PGs, and mitogen or antigen stimulation of human or murine lymphocytes leads to their enhanced release⁵⁻⁸. They are also released from various human and animal tumours^{9,10}. Thus, as cells of the immune system are both sensitive to and sources of PGs, these factors may be important as physiological immune regulators. For example, PGs of the E series are capable of inhibiting many effector functions⁹⁻¹⁵. They have also been shown to inhibit the development of the humoral response^{16,17}. Although they inhibit the proliferative response to mitogens^{18,19}, little is known about their effects on the development of the cell-mediated immune response to antigens. The data summarized here implicate PGs, thromboxane A₂ (TXA₂) and prostacyclin (PGI₂) in the regulation of cellular immune responses at the inductive phase. Some of these data have been reported in abstract form²⁰.

A dose-response relationship was observed when various numbers of X-ray irradiated P815 mastocytoma cells were cultured with a constant number (5×10^6) of allogeneic C57BL/6 spleen cells. The cell-mediated immunity (CMI) was monitored by ³H-thymidine incorporation and anti-target cell lytic activity (% of specific ⁵¹Cr release) on day 4 in culture. The ³H-thymidine incorporation into unstimulated lymphocytes was $1,664 \pm 801$ c.p.m. and increasing the number of stimulator cells from 1×10^5 to 5×10^5 led to an increase in both ³H-thymidine incorporation ($8,576 \pm 3,928$ to $61,998 \pm 14,079$ c.p.m.) and cytotoxic activity (% specific ⁵¹Cr release 22.7 ± 7.7 to 52.5 ± 6.8 , effector: target cell (E:T) = 50:1). A further increase of stimulator cells to 1×10^6 decreased the levels of both these parameters to those of the unstimulated culture. The ³H-thymidine uptake into the cells was only a measure of the proliferation of the lymphocytes after antigenic stimulation. The ultimate proof of immunological significance of the stimulation is the production of cytolytic effector cells.

Table 1a shows that addition of PGE₁ or PGE₂ on day 0 of culture had an inhibitory effect on ³H-thymidine incorporation on day 4 of culture. At this time the response was maximal. The ³H-thymidine incorporation into the lymphocytes was 24% and 38% of the control in the presence of 30 nM PGE₁ and 30 nM PGE₂, respectively. The agents at these concentrations had no effect on the unstimulated lymphocytes or on the ³H-thymidine incorporation when added at the time of assay. As indicated by the ⁵¹Cr release assay (Table 1b), 30 nM of PGE₁ or PGE₂ inhibited the development of the CMI by 50%. PGE at the concentrations tested had no effect on the lytic activity of effector cells generated *in vitro* when added at the time of the ⁵¹Cr release assay. This lack of activity on *in vitro*-generated effector cells compared with the effect on *in vivo*-generated effector cells has recently been reported¹⁵. The concentration of PGE found in inflammatory fluid⁷ and in mitogen- or antigen-stimulated cultures^{5,6} is in the range of 3–300 nM. Thus, the finding that 30 nM PGE₁ or PGE₂ could inhibit sensitization of lymphocytes is consistent with the hypothesis that, at physiological levels, these agents act as negative-feedback regulators.

Kinetic experiments indicated that PGE₁ affects only the magnitude of the CMI and does not cause a shift in the responses. When PGE₁ was added at various intervals after culture initiation, it was found to act during the inductive phase of the immune response. PGE₁ was active when added to spleen cells as long as 20 h before and up to 24 h after the addition of stimulator cells, but when added 48 h after culture initiation, it was not effective. It has recently been reported that if human peripheral blood lymphocytes are cultured for 24 h before being stimulated by phytohaemagglutinin, their proliferation is no longer inhibited by PGs²¹. However, this study shows that the antigenic stimulation of murine spleen lymphocytes which had been preincubated for 20 h, can still be modulated by PGE₁. In separate experiments, when PGE₁ was in spleen cell culture for 20 h and was removed by washing before the addition of stimulator cells, little inhibition was observed (data not shown). Thus, these data indicate the reversibility of the effect and the fact that PGE₁ seems to have a relatively long half life in spleen cell cultures. The effect of PGE₁ was also negated if the agent was removed by washing the cells within 8 h after initiation of the culture. By 48 h the effect of PGE₁ was irreversible. In summary, the effects of PGE on the CMI response affect the early period of the induction phase of the response.

The results shown in Fig. 1 indicate that indomethacin, an irreversible inhibitor of cyclooxygenase, the enzyme that catalyses the conversion of arachidonic acid to the endoperoxides PGG₂ and PGH₂ (which are common intermediates for TXA₂, PGE₂, PGF_{2 α} , PGI₂ and other PGs)²², augmented

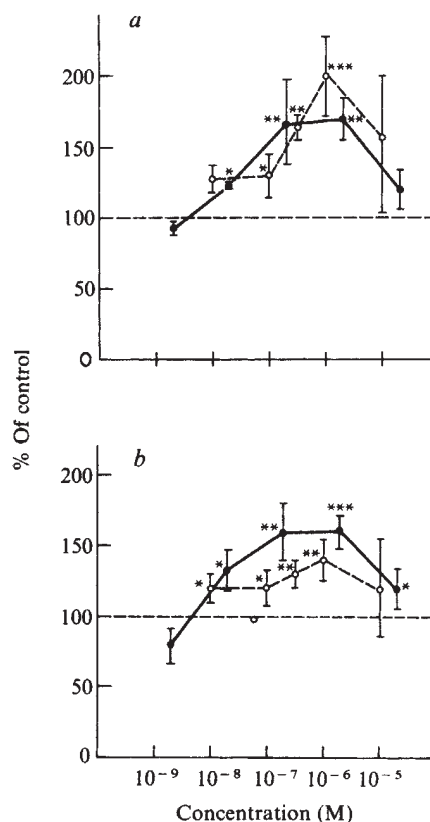


Fig. 1 Effect of indomethacin and Ro 20-5720 on the development of the CMI. *a*, Thymidine incorporation; *b*, cytotoxicity. The results were expressed in terms of the non-drug-treated control normalized to 100. The control values for the thymidine incorporation for indomethacin and Ro-5720 were $7,372 \pm 1,370$ and $15,341 \pm 3,115$ c.p.m., respectively. The control values for ⁵¹Cr release were 24.3 ± 3.2 and 23.4 ± 2.6 c.p.m., respectively. Each value represents the mean % of control $\pm$ s.d. ($n=4$). * $P < 0.005$, ** $P < 0.001$ and *** $P < 0.001$.

Table 1 Effects of prostaglandins E₁ and E₂ on ³H-thymidine incorporation into spleen lymphocytes* (a) and the cytolytic activity of effector cells (b)

a	PGE concentration (nM)	Nonstimulated cultures (c.p.m. ± s.d.) [†]		Stimulated cultures (5 × 10 ⁵ P815x) (c.p.m. ± s.d.) [†]			
		PGE ₁	PGE ₂	PGE ₁	PGE ₂		
(1) Inductive phase [‡]							
	0	1,073 ± 171		46,398 ± 2,874			
	0.3	1,280 ± 91 (120) [§]	1,628 ± 360 (181)	46,992 ± 5,912 (101)	38,832 ± 1,987 (84) ^{**}		
	3	1,299 ± 166 (120)	1,530 ± 220 (142)	30,585 ± 2,440 (66) [¶]	34,048 ± 1,766 (73) [¶]		
	30	1,110 ± 363 (102)	1,220 ± 110 (114)	11,090 ± 536 (24) [*]	17,512 ± 1,761 (38) [*]		
	3,000	712 ± 88 (66)	790 ± 145 (74)	5,668 ± 364 (12) [*]	8,224 ± 1,096 (18) [*]		
(2) Effector phase ^{††}							
	3	ND	ND	44,439 ± 3,441 (96)	43,285 ± 2,849 (93)		
	30	ND	ND	45,057 ± 3,034 (96)	44,800 ± 2,717 (97)		
	3,000	1,348 ± 325 (126)	1,281 ± 265 (119)	49,252 ± 5,796 (106)	46,173 ± 4,259 (100)		
b	PGE concentration (nM)	% Specific ⁵¹ Cr release ± s.d. [†] (E:T ratios)					
		PGE ₁			PGE ₂		
		50:1	25:1	12.5:1	50:1	25:1	12.5:1
(1) Inductive phase [‡]							
	0	53.0 ± 0.7	37.7 ± 2.4	23.0 ± 1.4	53.0 ± 0.7	37.7 ± 2.4	23.0 ± 1.4
	0.3	50.9 ± 3.7 (96) [§]	36.2 ± 5.1 (96)	19.5 ± 0.9 (85)	49.4 ± 1.4 (93)	37.9 ± 1.6 (100)	21.3 ± 1.6 (93)
	3	45.6 ± 2.0 (86) ^{**}	31.6 ± 2.7 (84)	18.0 ± 1.4 (78)	47.5 ± 3.6 (90)	33.9 ± 3.2 (90)	19.8 ± 1.5 (86)
	30	24.3 ± 0.7 (46) [*]	14.9 ± 1.4 (40)	6.9 ± 2.3 (30)	32.6 ± 2.0 (62) [¶]	19.7 ± 1.0 (52)	12.3 ± 0.7 (53)
	3,000	10.0 ± 1.1 (19) [*]	5.0 ± 1.0 (10)	1.3 ± 0.8 (6)	16.4 ± 2.4 (31) [*]	10.0 ± 1.9 (27)	4.4 ± 1.2 (19)
(2) Effector phase							
	3,000	49.1 ± 2.7 (93)	33.8 ± 1.5 (90)	ND	50.3 ± 1.1 (95)	33.5 ± 1.9 (89)	ND
	30,000	47.2 ± 0.6 (89)	32.7 ± 1.0 (87)	ND	46.8 ± 1.4 (88)	32.3 ± 0.6 (86)	ND

Primary immunization cultures with C57BL/6 mouse spleen cells as responder cells and P815 tumour cells as stimulator cells were established according to published procedures³⁰. Briefly, cultures were maintained in RPMI 1640 medium supplemented with 2 mM L-glutamine, 25 mM HEPES (pH 7.45), 3×10^{-5} M mercaptoethanol, 10% heat-inactivated fetal calf serum (FCS, Gibco), 100 μ g ml⁻¹ streptomycin, 100 U ml⁻¹ penicillin and 100 μ g ml⁻¹ gentamycin. A suspension of 5×10^6 cells in 1.9 ml of supplemented medium was added to 35-mm tissue culture dishes and the cells were incubated at 37 °C in a 5% CO₂ atmosphere. Stimulator cells were X-ray irradiated (4,000R, P815x cells) and test agents were added in 0.1 ml medium to the spleen cell suspension. In some cases, 95% ethyl alcohol (EtOH) was used as initial solvent, but the final concentration did not exceed 0.01% which was shown not to affect the assays. The same volume of medium was added to control cultures. In these conditions, the cytotoxicity of effector cells (E) that developed was proportional to the number of stimulating cells which were varied from 5×10^5 to 2.5×10^6 . To measure thymidine incorporation, microcultures were incubated in flat-bottom Microtest plates (Linbro) at 37 °C and 5% CO₂ in air. The microcultures consisted of 0.1 ml cell suspension from day 4 culture and 0.1 ml of RPMI medium and 50 μ l (1 μ Ci) of [*Me*-³H]thymidine (specific activity 6.7 Ci mmol⁻¹). After 4 h the cultures were collected on glass filter strips with an automatic cell collector. The filters were washed with saline. They were dried and counted in scintillation fluid in a Beckman scintillation counter. *In vitro* effector cells were collected after day 4 and unless otherwise stated, were centrifuged, washed and resuspended in 0.5 ml of medium supplemented with 5% FCS. Cell viability was determined by trypan blue exclusion and was unaffected by the drug treatments. The density of viable effector cells was adjusted to 1×10^7 cells ml⁻¹. ⁵¹Cr-labelled P815 target cells (T) were prepared according to published procedures³¹, and their cell density adjusted to 2×10^5 cells ml⁻¹. Labelled target cells (2×10^4) in 0.1 ml were added to 0.1 ml of effector cells in duplicate 12 × 75-mm plastic tubes (Falcon). The number of effector cells was adjusted to give different E:T ratios—50:1, 25:1 and 12.5:1. The preparations were incubated for 4 h at 37 °C in a humidified CO₂ incubator and the reaction was stopped by adding 2 ml of cold RPMI 1640 medium. The percentage release of radioactive chromium was calculated as follows:

$$\% \text{ } ^{51}\text{Cr release} = \frac{\text{c.p.m. in supernatant}}{\text{c.p.m. in supernatant} + \text{c.p.m. in pellet}} \times 100$$

The per cent specific ⁵¹Cr release is obtained by subtracting the % ⁵¹Cr released from labelled targets in the presence of nonsensitized spleen cells from the value obtained in the presence of sensitized spleen cells. All experiments have been reproduced at least twice.

* Spleen lymphocytes (5×10^6) were cultured with 5×10^5 P815x cells for 4 days.

[†] These values represent the mean ± s.d. where *n* = 3.

[‡] The final concentration of either PGE₁ or PGE₂ added at the time of initiation of the culture.

[§] The numbers in parentheses represent per cent of control without PG.

^{||} The statistical significance which was determined by the Student's two-tailed *t*-test is indicated by: ||, *P* < 0.05; ¶, *P* < 0.01; and *, *P* < 0.001.

^{††} Prostaglandins were added at the time of assay on day 4. ND, not done.

³H-thymidine incorporation and lytic activity in a concentration-dependent manner. The optimal concentration was 3×10^{-7} M (0.1 μ g ml⁻¹). Ro 20-5720 (*d*,1-6-chloro- α -methyl-carbazole-2-acetic acid), a reversible inhibitor of cyclo-oxygenase¹⁷, also augmented the CMI (optimal concentration 10^{-6} M). Inhibitors of PG biosynthesis have been extensively studied (for a recent review, see ref. 23). Inhibitors of cyclo-oxygenase have been shown previously to inhibit tumour

growth¹⁶, enhance humoral responses¹⁷ and augment mitogen- or antigen-induced blastogenesis^{18,19}.

In addition to the PGs, PGI₂ and TXA₂ are also released by macrophages and lymphocytes^{8,23-25}. PGI₂ (3×10^{-4} M) inhibited the cytolytic activity of effector cells from immunized mice by 20% (*P* < 0.02), whereas at concentrations from 3×10^{-10} M to 3×10^{-6} M it showed no effect on the development of the CMI in culture. This lack of activity may be due to the short

Table 2 Effects of exogenous PGE₂ on the CMI in the presence or absence of inhibitors of prostaglandin synthesis

Inhibitors	Thymidine incorporation			Per cent of control*			Cytotoxicity		
				PGE ₂ (nM)					
	0	3	30	0	3	30	0	3	30
0	100	69 ± 6 [§]	30 ± 3 [§]	100	80 ± 12 [†]	48 ± 13 [§]			
Indomethacin (0.1 µg ml ⁻¹)	141 ± 13 [§]	76 ± 8 [†]	30 ± 8 [§]	125 ± 9 [†]	94 ± 23	37 ± 7 [§]			
Imidazole (IDZ) (200 µg ml ⁻¹)	55 ± 11 [§]	39 ± 10 [§]	16 ± 2 [§]	49 ± 16 [‡]	38 ± 11 [§]	12 ± 8 [§]			
Tranlycypromine (Tranyl) (25 µg ml ⁻¹)	144 ± 23 [‡]	87 ± 10	32 ± 7 [§]	133 ± 4 [§]	127 ± 8 [‡]	75 ± 16			
IDZ/Tranyl (200 µg 25 µg ml ⁻¹)	59 ± 8 [§]	35 ± 3 [§]	13 ± 1 [§]	87 ± 21	58 ± 25 [‡]	18 ± 20 [§]			

Spleen cell cultures were exposed to 3.5×10^5 P815x cells and indomethacin, imidazole, tranlycypromine or PGE₂ alone or in the indicated combinations.

* The results were expressed as per cent of non-drug-treated control culture response. The control values for thymidine incorporation and % specific ⁵¹Cr release were $40,197 \pm 14,977$ and 49.5 ± 5.7 (E:T ratio 50:1), respectively. Each value represents the mean % of control of four experiments $\pm$ s.d.

[†] $P < 0.05$, [‡] $P < 0.01$, [§] $P < 0.001$.

half life of PGI₂ ($t_{1/2} = 3-5$ min) in culture conditions²⁶. The half life of TXA₂ is also extremely short ($t_{1/2} = 30$ s)²⁷. Knowing these facts, tranlycypromine, an inhibitor of PGI₂ synthetase (ID₅₀ concentration $160 \mu\text{g ml}^{-1}$)²⁶ and imidazole, an inhibitor of thromboxane synthetase (ID₅₀ concentration 8 mM)^{27,28}, were tested to assess their effect on the CMI. Note that the effects of these two agents are specific for the respective enzymes and that at the concentrations tested here they do not affect other enzymes of arachidonic acid metabolism. That they may affect other enzyme systems cannot be ruled out. In fact, imidazole is known to stimulate phosphodiesterase²⁸ and such stimulation reduces cyclic AMP pools and results in either no effect or augmented CMI development (K.H.L., unpublished

results). The data (Fig. 2) show that imidazole (ID₅₀ 3 mM or $200 \mu\text{g ml}^{-1}$) inhibited the development of the CMI whereas tranlycypromine (0.2 mM or $25 \mu\text{g ml}^{-1}$) augmented it in a concentration-dependent manner. When used in combination, imidazole and tranlycypromine were antagonistic in their effect on the CMI. There is, however, an apparent constant minimal per cent reduction of the CMI caused by imidazole regardless of the concentration of tranlycypromine used, and similarly, the augmentation with tranlycypromine in the presence of different concentrations of imidazole remains constant at a certain minimal level. This may reflect the fact that a blockage of TXA₂ synthesis by imidazole leads to a lower TXA₂ pool, but also a consequence of this blockage could be increased synthesis of PGI₂ and PGE₂. In the presence of imidazole, the effect of tranlycypromine is less effective and vice versa. This may suggest that, apart from inhibition of PGI₂ synthesis by tranlycypromine, some of the action may be due to the possible increased production of TXA₂.

Table 2 summarizes the effects of exogenous PGE₂ on the CMI in the presence or absence of inhibitors of PG synthesis. The effect of indomethacin was reversible by PGE₂, indicating that PGE₂ is a negative mediator of the CMI development. The effects of imidazole and PGE₂ were additive. However, 3 nM of PGE₂ did not effectively reverse the augmented CMI resulting from the addition of tranlycypromine. More interestingly, the effect of tranlycypromine on the ³H-thymidine incorporation was abrogated by 30 nM of PGE₂, but its effect on the cytotoxic activity was not completely reversed ($P < 0.01$). This may suggest that PGE₂ is less effective in reversing the action of TXA₂ in the presence of a lower level of PGI₂ and TXA₂; PGE₂ or PGI₂ may be affecting different subsets of lymphocytes. In the presence of imidazole and tranlycypromine, the effect of PGE₂ was, as in the presence of indomethacin, due to the lower PGI₂ and TXA₂ pools. This seems to indicate that PGI₂ and TXA₂ are more potent than PGE₂ in modulating the development of CMI.

Three implications may be derived from these results: (1) PGI₂ may be a negative (inhibitory) regulator and TXA₂ a positive (augmentative) regulator of the CMI development. (2) Augmentation of the CMI by tranlycypromine may be a consequence of the removal of PGI₂ or of a channelling of PGH₂ into TXA₂ and other PGs. The converse of this argument may be applied to explain the lower CMI response following inhibition of TXA₂ synthesis by imidazole. (3) The relative concentrations of PGI₂, TXA₂ and PGs may determine the magnitude of the immune response.

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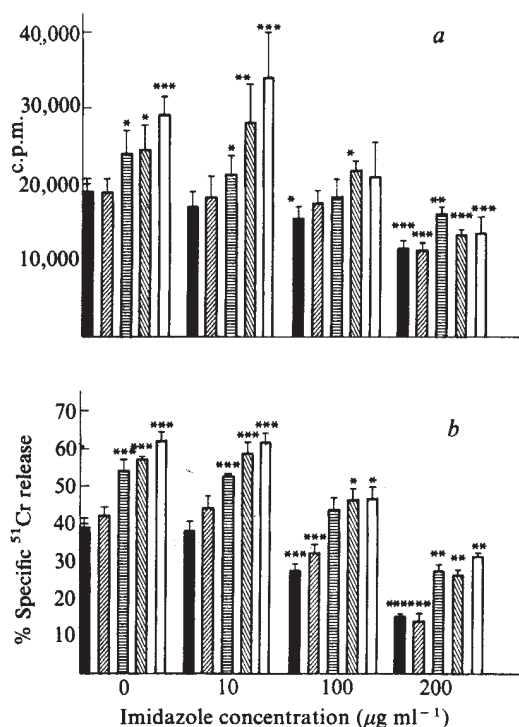


Fig. 2 Effect of imidazole or tranlycypromine alone or in combination on the development of the CMI. *a*, Thymidine incorporation; *b*, cytotoxicity. The results were expressed as mean $\pm$ s.d. ($n = 4$). *, $P < 0.005$, **, $P < 0.01$ and ***, $P < 0.001$. The concentrations of tranlycypromine were: ■, $0 \mu\text{g ml}^{-1}$; □, $1 \mu\text{g ml}^{-1}$; ▨, $5 \mu\text{g ml}^{-1}$; ▩, $10 \mu\text{g ml}^{-1}$; and ▤, $25 \mu\text{g ml}^{-1}$.

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1. Bourne, H. R. *et al. Science* **184**, 19–28 (1974).
2. Pelus, L. M. & Strasser, H. R. *Life Sci.* **20**, 903–914 (1977).
3. Lewis, G. P. *J. reticuloendothel. Soc.* **22**, 389–402 (1977).
4. Goodwin, J. S. & Webb, D. R. *Clin. Immun. Immunopath.* **15**, 106–122 (1980).
5. Ferraris, V. A. & DeRubertis, F. R. *J. clin. Invest.* **54**, 378–386 (1974).
6. Webb, D. R. & Osheroff, P. L. *Proc. natn. Acad. Sci. U.S.A.* **73**, 1300–1304 (1976).
7. Youlten, L. J. F. & McCall, E. in *Prostaglandins in Hematology* (eds Silver, M. J., Smith, J. B. & Kocsis, J. J.) 87–90 (Spectrum, New York, 1977).
8. Humes, J. L. *et al. Nature* **269**, 149–151 (1977).
9. Jaffe, B. M. *Prostaglandins* **6**, 453–461 (1977).
10. Easty, G. C. & Easty, D. M. *Cancer Treat. Rev.* **3**, 217–225 (1976).
11. Henney, C. S., Bourne, H. R. & Lichtenstein, L. M. *J. Immun.* **108**, 1526–1534 (1972).
12. Strom, T. B. *et al. J. exp. Med.* **138**, 381–383 (1973).
13. Gorden, D., Bray, M. A. & Morley, J. *Nature* **262**, 401–402 (1976).
14. Schultz, R. M., Pavlidis, N. A., Stylos, W. A. & Chirigos, M. A. *Science* **202**, 320–321 (1978).
15. Plaut, M. J. *Immun.* **123**, 692–701 (1979).
16. Plescia, O. J., Smith, A. H. & Grinwich, K. *Proc. natn. Acad. Sci. U.S.A.* **72**, 1848–1851 (1975).
17. Webb, D. R. & Nowowiejski, I. *Cell Immun.* **33**, 1–10 (1977).
18. Goodwin, J. S., Bankhurst, A. D. & Messner, R. P. *J. exp. Med.* **146**, 1719–1734 (1977).
19. Webb, D. R. & Nowowiejski, I. *Cell Immun.* **41**, 72–85 (1978).
20. Leung, K. H. & Mihich, E. 63rd A. Meet. Fedn Am. Soc. exp. Biol., Dallas, Texas (1–10 April, 1979); 1st Int. Conf. Immunopharmac., Brighton (29 July–1 August, 1980).
21. Goodwin, J. S., Messner, R. P. & Peake, G. T. *J. clin. Invest.* **62**, 753–760 (1978).
22. Samuelsson, B. *et al. A. Rev. Biochem.* **47**, 997–1029 (1978).
23. Vane, J. R. *Adv. Prostaglandin Thromboxane Res.* **4**, 27–44 (1978).
24. Brune, K., Glatt, M., Kalin, H. & Peskar, B. A. *Nature* **274**, 261–263 (1978).
25. Morley, M. A., Bray, M. A., Jones, R. W., Nuteven, D. H. & Van Doryn, D. A. *Prostaglandins* **17**, 730–746 (1979).
26. Gryglewski, R. J., Bunting, S., Moncada, S., Flower, R. J. & Vane, J. R. *Prostaglandins* **12**, 685–713 (1976).
27. Moncada, S. *et al. Prostaglandins* **13**, 611–618 (1977).
28. Parker, C. W., Stevenson, W. F., Huler, M. G. & Kelly, J. P. *J. Immun.* **122**, 1572 (1979).
29. Needleman, P. *et al. Prostaglandins* **14**, 897–907 (1977).
30. Cerottini, J.-C., Engers, H. D., MacDonald, H. R. & Brunner, K. T. *J. exp. Med.* **140**, 703–717 (1974).
31. Mawas, C., Carey, T. & Mihich, E. *Cell Immun.* **6**, 243–260 (1973).

Platelet-dependent stimulation of prostacyclin synthesis by platelet-derived growth factor

Shaun R. Coughlin*, Michael A. Moskowitz*†, Bruce R. Zetter‡, Harry N. Antoniades§ & Lawrence Levine||

* Laboratory of Neural and Endocrine Regulation, Department of Nutrition and Food Science, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

† Section of Neurology, Department of Medicine, Peter Bent Brigham Hospital, Harvard Medical School, Boston, Massachusetts 02115

‡ Department of Surgery, Children's Hospital Medical Center, Harvard Medical School, Boston, Massachusetts 02115

§ Center for Blood Research, Department of Nutrition, Harvard School of Public Health, Boston, Massachusetts 02115

|| Department of Biochemistry, Brandeis University, Waltham, Massachusetts 02154

Prostacyclin (PGI₂), an unstable metabolite of arachidonic acid synthesized by vascular endothelial and smooth muscle cells, is a potent vasodilator and endogenous inhibitor of platelet aggregation^{1–10}. Regulation of PGI₂ synthesis by the vessel wall is not well understood^{11–16}. We have investigated the possibility that a product released from platelet granules during degranulation might modify vessel wall PGI₂ biosynthesis. We report here that a non-dialysable, platelet-dependent factor in serum dramatically stimulates PGI₂ synthesis by cultured bovine aortic endothelium, aortic smooth muscle, and adrenal capillary endothelium. Platelet-derived growth factor (PDGF), a releasable peptide contained within platelet alpha granules^{17–20}, stimulates PGI₂ synthesis by the above cell types as much as 100-fold. The concentrations of PDGF required to produce these effects are below the level reported in normal human serum¹⁸. We postulate that *in vivo* released PDGF may increase vessel wall PGI₂ production as part of a negative feedback mechanism controlling platelet aggregation.

† To whom correspondence should be addressed.

Endothelial cells were cultured from bovine aorta by a modification of previously published techniques (see legend to Table 1)^{21–23}. These cells formed confluent monolayers and exhibited light-microscopic and ultrastructural characteristics consistent with endothelium^{24–26}. They also contained the endothelial cell marker factor VIII antigen²⁴; immunofluorescent studies revealed specific granular staining concentrated in the perinuclear region (anti-bovine factor VIII provided by J. Brown, University of California, San Diego). Bovine aortic smooth muscle cells were cultured as described elsewhere²⁷. These appeared spindle-shaped, grew in multilayers with the characteristic 'hill and valley' pattern, and contained prominent myofilamentous bundles characteristic of smooth muscle. Culture and characterization of bovine adrenal capillary endothelium has been reported elsewhere²⁸. The capillary endothelial cells used in this study were cloned from single cells and used between passages eight and twelve. Bovine aortic endothelial and smooth muscle cell cultures between passages one and five were used.

Cells in culture were incubated with vehicle or treatment as described in Table 1. Of the total cyclo-oxygenase products made in culture, aortic endothelium made 95% 6-keto prostaglandin F_{1α} (6 KF_{1α}), capillary endothelium 80%, and aortic smooth muscle 73% 6 KF_{1α}. In descending order, lesser amounts of PGF_{2α}, PGE₂, PGD₂ and thromboxane B₂ (TXB₂) were also made. For comparison, aortic strips *in vitro* made about 96% 6KF_{1α} and brain microvessels made 80% (ref. 29).

Serum has been reported to stimulate prostaglandin synthesis by MC5-5 fibroblasts in culture³⁰. In preliminary experiments we demonstrated that human or bovine serum also potently stimulated prostaglandin and prostacyclin synthesis in cells of vascular origin, bovine aortic endothelium and smooth muscle. To assess the platelet's contribution to this activity, platelet-rich plasma-derived serum (PRS) and platelet-poor plasma-derived serum (PDS) were prepared (see Table 1) and tested for their ability to stimulate PGI₂ synthesis in culture. The data shown in Table 1 indicate that at least 80% of the stimulatory activity of serum seems to be platelet-dependent. (The stimulation of PGI₂ synthesis by PDS may be due to PDGF released during plasma preparation, thrombin³¹, bradykinin, angiotensin II (ref. 32), or other as yet unidentified compounds.) Preparations with higher specific activity than PRS have been obtained by heating (100 °C for 2.5 min) and extracting platelets into 1.0 M NaCl. (Specific activity is defined as ng 6KF_{1α} accumulated per mg of added protein³³.) These data point to the platelet as a major source of PGI₂-stimulating activity found in serum. This platelet-dependent activity was nondialysable, stable at 56 °C, and at least partially stable at 100 °C—properties similar to those of platelet-derived growth factor¹⁷. Because PDGF was recently proposed as the component in serum responsible for phospholipase A₂ activation in Swiss mouse 3T3 fibroblasts³⁴, the ability of PDGF to stimulate PGI₂ synthesis in endothelial and smooth muscle cells was assessed.

PDGF was purified¹⁷ from human platelets and incubated with cultured bovine aortic endothelial, smooth muscle, and adrenal capillary endothelial cells (see Table 2). The PDGF used in this study migrated as a single band on polyacrylamide electrophoresis with an apparent molecular weight of 35,000 in non-reducing conditions—after reduction with 5% (v/v) 2-mercaptoethanol PDGF migrated as two bands with apparent MW 13,000 and 17,000. As shown in Table 2, PDGF stimulated dose-dependent biosynthesis of PGI₂. When added at a concentration of 8.0 ng ml⁻¹, PDGF stimulated the synthesis of PGI₂ by 74-, 84- and 15-fold in aortic endothelial, smooth muscle, and capillary endothelial cell cultures, respectively. Followed over a 24-h period, 6KF_{1α} accumulated in an essentially linear manner in these cultures. To evaluate the reversibility of PDGF action, cultures were washed five times with 2.5 ml M199 after incubation for 6 h with PDGF (8 ng ml⁻¹) and the medium replaced with M199 plus vehicle only. After washing, the rate of 6KF_{1α} accumulation returned to that of the unstimulated cultures. Thus, PDGF-induced stimulation

required the continued presence of PDGF in the medium. Similar results were obtained with PRS. In addition to stimulating PGI₂ synthesis, PDGF stimulated PGF_{2α} synthesis by the above cell types, with a similar dose response. This suggests that PDGF increases prostaglandin production either by stimulating the cyclo-oxygenase, or, more likely, by increasing arachidonate availability to the cyclo-oxygenase through increasing phospholipid deacylation. In fact, PDGF activation of phospholipase A₂ has recently been reported³⁴. Serum stimulates prostaglandin synthesis in a similar manner in these and other cells^{30,34}.

Table 1 Platelet-dependent stimulation of PGI₂ synthesis

	Control	PRS	PDS
Aortic endothelium			
6KF _{1α} (ng ml ⁻¹)	4.1 ± 0.44 ¹	496 ± 87 ²	123 ± 17 ³
Cells per plate (×10 ⁻⁵)	16.1 ± 0.59 ¹	20.1 ± 1.1 ²	19.3 ± 0.47 ²
Aortic smooth muscle			
6KF _{1α} (ng ml ⁻¹)	<0.007 ¹	9.84 ± 0.24 ²	0.85 ± 0.041 ³
Cells per plate (×10 ⁻⁵)	3.60 ± 0.10 ¹	5.15 ± 0.21 ²	4.18 ± 0.44

Platelet-rich plasma derived serum (PRS) and platelet-poor plasma-derived serum (PDS) were incubated with vascular cells in culture as described below. Bovine aortic endothelial cells were cultured by a modification of existing techniques²¹⁻²³. Briefly, descending thoracic aortas were excised aseptically from week-old calves within 15 min of death and transported in calcium-magnesium-free Dulbecco's phosphate-buffered saline (PBS) with antibiotics (ABX) (penicillin 50 µg ml⁻¹; streptomycin 50 µg ml⁻¹; neomycin 100 µg ml⁻¹ and tylocine 60 µg ml⁻¹). The adventitia was removed, the lumen washed with 500 ml of PBS, the intercostals and distal end clamped and the lumen filled with 1 mg ml⁻¹ collagenase (Worthington Biochemical, 135 U per mg) in Hank's balanced salt solution (Gibco). With the proximal and distal ends occluded, the vessel was incubated by immersion in PBS at 37 °C for 20 min. The collagenase solution was collected, the lumen washed gently four times with culture medium and the washes and collagenase solution were pooled and spun at 100g for 5 min. The pellet was resuspended in culture medium and plated on tissue culture dishes pretreated with gelatin²⁸ and fibronectin (Collaborative Research) at 1 µg per cm². Culture medium consisted of 1:1 mixture of alpha MEM and Dulbecco's modified Eagle's medium (Gibco) with 20% calf serum (Microbiological Associates), 2 mM added L-glutamine, 25 µg ml⁻¹ endothelial cell growth supplement (Collaborative Research) and ABX. Bovine aortic smooth muscle and adrenal capillary endothelial^{27,28} cells were plated 48 h before the start of an experiment at 1–4 × 10⁵ cells per 35 mm tissue culture dish in the appropriate growth conditions for each cell type. At the start of each experiment, the plates were washed twice with 2.5 ml of M199 (Medium 199 (Gibco) with 2 mM added L-glutamine), 2.5 ml of M199 plus vehicle or treatment and the plates returned to the incubator (5% CO₂/95% air, 37 °C) for the time required. After incubation, the medium was removed and transferred to tubes containing indomethacin (final concentration 4 µM) and frozen until required for assay. Cell numbers were determined using a Coulter Counter (Coulter Electronics) after washing with PBS, briefly treated with trypsin-EDTA solution (Gibco), and suspended in 2% formaldehyde in PBS. PGI₂ and TXA₂ synthesis was assessed by measuring the accumulated levels of their respective stable products¹⁵, 6KF_{1α} and TXB₂, PGF_{2α}, PGE₂, PGD₂, TXB₂ and 6KF_{1α} concentrations were determined by radioimmunoassay of the incubation medium using techniques described elsewhere²⁵. The specificity of this assay system is such that results obtained by direct radioimmunoassay (RIA) of the incubation media are similar to those obtained by first separating the prostaglandins in the media by high performance liquid chromatography and then measuring each fraction by RIA^{36,37}. PRS and PDS were prepared by a modification of published techniques²⁰. Blood was drawn by venipuncture from normal volunteers into 1/10 volume 3.82% sodium citrate. The anticoagulated whole blood was spun at 100g for 20 min, the platelet-rich plasma (PRP) collected and divided into two portions. One half was clotted by the addition of 1 M CaCl₂ (1 ml per 50 ml PRP) followed by incubation for 3 h at 37 °C. The rest was spun at 2,000g for 15 min, the supernatant collected and the platelet pellet resuspended in 1 M NaCl for future extraction. The supernatant was further spun at 20,000g for 30 min to remove any residual platelets, then clotted with CaCl₂ as above. The resulting PRS and PDS were allowed to stand at 4 °C overnight, centrifuged at 20,000g for 30 min, dialysed (MW cutoff 3,000) against 500 volumes of 145 mM NaCl, 4 mM KCl, 3 mM CaCl₂, and 1.5 mM MgCl₂, measured out and frozen for later use. Serotonin was measured in pre-dialysis samples of PRS and PDS by liquid chromatography with electrochemical detection³⁸ as an index of platelet degranulation. Serotonin levels in the PRS and PDS used in this study were >700 nM and <1 nM, respectively. 500 µl of PRS or PDS were added directly to culture dishes containing 2.0 ml of M199. Twenty-four hour incubations were carried out and culture fluids assayed. 6KF_{1α} was not detectable in the dialysed PRS or PDS. Indomethacin (4 µM) inhibited 6KF_{1α} synthesis in all cultures by over 99%. Data were analysed by one-way analysis of variance; multiple comparisons were made by the significant difference method³⁹. Values shown are mean ± s.e.m. (n = 5). Both PRS and PDS stimulated PGI₂ biosynthesis by bovine aortic endothelial and smooth muscle cells, but PDS was at least five times less active in this capacity. Full recovery of PGI₂ stimulating activity was achieved by recombining platelets and platelet-poor plasma before clotting. Means with different superscripts were significantly different (P < 0.01).

Table 2 PDGF stimulates PGI₂ synthesis

Cell type	PDGF (ng ml ⁻¹)	6KF _{1α} (ng ml ⁻¹)	Cell number per plate (×10 ⁻⁵)
Aortic endothelium	Vehicle	0.337 ± 0.007 ¹	12.8 ± 0.81
	0.08	0.518 ± 0.072 ²	15.9 ± 0.86
	0.80	2.370 ± 0.133 ³	11.3 ± 0.50
	8.00	25.000 ± 5.000 ⁴	13.8 ± 1.20
Aortic smooth muscle	Vehicle	<0.007 ¹	3.71 ± 0.14 ¹
	0.08	0.013 ± 0.007 ²	4.58 ± 0.20 ¹
	0.80	0.079 ± 0.015 ³	5.76 ± 0.07 ¹
	8.00	0.593 ± 0.077 ⁴	8.51 ± 0.68 ²
Capillary endothelium	Vehicle	0.056 ± 0.015 ¹	5.52 ± 0.54
	0.08	0.035 ± 0.003 ¹	5.19 ± 0.25
	0.25	0.045 ± 0.001 ¹	4.60 ± 0.32
	0.80	0.059 ± 0.010 ¹	4.81 ± 0.29
	2.53	0.213 ± 0.018 ²	4.30 ± 0.51
	8.00	0.813 ± 0.059 ³	5.30 ± 0.78

Platelet PDGF was purified¹⁷, added to 1 M NaCl in polypropylene tubes and frozen for later use. PDGF was thawed immediately before use, diluted, if necessary, in 1 M NaCl, and added to 35 mm culture vessels containing 2.5 ml M199. Controls received 25 µM NaCl per plate, the maximum amount of NaCl added to any PDGF-treated culture. This amount of NaCl did not affect prostaglandin production. Twenty-four hour incubations were carried out and culture fluids assayed as described in the text. Prostaglandins were not detected in culture fluid from plates incubated with PDGF (8.0 ng ml⁻¹) without cells. Indomethacin inhibited PDGF-stimulated PGI₂ synthesis by 99.8%. Data were analysed as described in the legend to Table 1. Means with different superscripts were significantly different (P < 0.01). Values shown are mean ± s.e.m. (n = 4).

As shown in Table 2, the well known mitogenic effects of PDGF were observed with smooth muscle cells but not with aortic or capillary endothelium. The prostacyclin-stimulating activity of PDGF, however, was seen with all three cell types. Thus the effects of PDGF on arachidonic acid metabolism were not always associated with its effects on cell division.

Thus both a platelet-dependent factor in serum and PDGF stimulate PGI₂ synthesis by vascular cells in culture. Both factors (1) appear to be platelet-derived; (2) are non-dialysable and at least partially heat stable (100 °C for 2.5 min); (3) stimulate cultures with a similar time course (linear 6KF_{1α} accumulation over 24 h); (4) act reversibly, and (5) stimulate PGI₂ synthesis most likely by increasing arachidonate availability for cyclo-oxygenase. We therefore suggest that PDGF may be the factor responsible for the platelet-dependent stimulation of PGI₂ synthesis by PRS. The level of PDGF reported in whole human serum is approximately 50 ng ml⁻¹ (ref. 18), well above the concentrations needed to stimulate PGI₂ synthesis *in vitro* in the present study. Thus PDGF may act locally to increase vascular PGI₂ production after its release *in vivo* at a site of platelet degranulation, as part of a negative feedback mechanism controlling platelet aggregation.

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- Moncada, S., Gryglewski, R., Bunting, S. & Vane, J. R. *Nature* **263**, 663–665 (1976).
- Bunting, S., Gryglewski, R., Moncada, S. & Vane, J. R. *Prostaglandins* **12**, 897–913 (1976).
- Gryglewski, R., Bunting, S., Moncada, S., Flower, R. & Vane, J. R. *Prostaglandins* **12**, 685–713 (1976).
- Johnson, R. A. *et al. Prostaglandins* **12**, 915–928 (1976).
- Baenziger, N. C., Dillender, M. J. & Majerus, P. W. *Biochem. biophys. Res. Commun.* **78**, 294–301 (1977).
- Weksler, B. B., Marcus, A. J. & Jaffe, E. A. *Proc. natn. Acad. Sci. U.S.A.* **74**, 3922–3926 (1977).
- Marcus, A. J., Weksler, B. B. & Jaffe, E. A. *J. biol. Chem.* **253**, 7138–7141 (1978).
- Ryan, J. W., Ryan, U. S., Habliston, D. & Martin, L. *Trans. Ass. Am. Physns* **91**, 343–350 (1978).
- MacIntyre, D. E., Pearson, J. D. & Gordon, J. L. *Nature* **271**, 549–551 (1978).
- Moncada, S. & Vane, J. R. *Fedn Proc.* **38**, 66–71 (1979).
- Moncada, S. & Vane, J. R. *Br. med. Bull.* **34**, 129–135 (1978).
- Marcus, A. J., Weksler, B. B. & Jaffe, E. A. *J. biol. Chem.* **253**, 7138–7141 (1978).
- Marcus, A. J. *Prog. Hemat.* **11**, 147–173 (1979).
- Needleman, P. *et al. J. clin. Invest.* **61**, 839–849 (1978).
- Baenziger, N. L., Becherer, P. R. & Majerus, P. W. *Cell* **16**, 967–974 (1979).

16. Smith, J. B. *et al.* *Circulation* **58**, 126 (1978).
17. Antoniadis, H. N., Scher, C. D. & Stiles, C. O. *Proc. natn. Acad. Sci. U.S.A.* **76**, 1809–1813 (1979).
18. Antoniadis, H. N. & Scher, C. D. *Proc. natn. Acad. Sci. U.S.A.* **74**, 1973–1977 (1977).
19. Kaplan, D. R., Chao, F. C., Stiles, C. D., Antoniadis, H. N. & Scher, C. D. *Blood* **53**, 1043–1052 (1979).
20. Ross, R., Glomset, J., Kariya, B. & Harker, L. *Proc. natn. Acad. Sci. U.S.A.* **71**, 1207 (1974).
21. Gimbrone, M. A. *Prog. Hemostasis & Thromb.* **3**, 1–28 (1976).
22. Jaffe, E. A., Hayer, L. W. & Wachman, R. L. *J. clin. Invest.* **52**, 2757–2764 (1973).
23. Booyse, F. M., Sedlak, B. J. & Rafelson, M. E. *Jr Thromb. Diath. haemorrh.* **34**, 825–839 (1975).
24. Jaffe, E. A., Nachman, R. C., Becker, C. G. & Minsk, C. R. *J. clin. Invest.* **52**, 2745–2756 (1973).
25. Gimbrone, M. A., Cotran, R. S. & Folkman, J. *J. Cell Biol.* **60**, 673–684 (1974).
26. Schwartz, S. M. *In Vitro* **14**, 966–980 (1978).
27. Ross, R. *J. Cell Biol.* **50**, 172–186 (1971).
28. Folkman, J., Haudenschild, C. & Zetter, B. *Proc. natn. Acad. Sci. U.S.A.* **76**, 5217–5221 (1979).
29. Maurer, P., Moskowitz, M., Levine, L. & Melamed, E. *Prostaglandins Med.* **4**, 153–161 (1980).
30. Hong, S. & Levine, L. *J. biol. Chem.* **251**, 5814–5816 (1976).
31. Weksler, B. B., Ley, C. & Jaffe, E. *J. clin. Invest.* **62**, 923–930 (1978).
32. Gryglewski, R., Korbut, R. & Splawinski, J. *Haemostasis* **8**, 294–299 (1979).
33. Lowry, O. H., Roseborough, N. J., Farr, A. L. & Randall, R. J. *J. biol. Chem.* **193**, 265–275 (1951).
34. Shier, W. T. *Proc. natn. Acad. Sci. U.S.A.* **77**, 137–141 (1980).
35. Levine, L. & Moskowitz, M. A. *Proc. natn. Acad. Sci. U.S.A.* **76**, 6632–6636 (1979).
36. Levine, L. & Alam, I. *Prostaglandins Med.* **3**, 295–304 (1979).
37. Alam, I., Ohuchi, K. & Levine, L. *Analyt. Biochem.* **93**, 339–345 (1979).
38. Reinhard, J. F., Moskowitz, M. A., Sved, A. F. & Fernstrom, J. D. *Life Sci.* (in the press).
39. Tukey, J. W. *Biometrics* **5**, 99–114 (1949).

Targeting to cells of fluorescent liposomes covalently coupled with monoclonal antibody or protein A

Lee D. Leserman, Jacques Barbet
& Francois Kourilsky

Centre d'Immunologie INSERM-CNRS de Marseille-Luminy,
Case 906, 13288 Marseille Cedex 2, France

John N. Weinstein

Laboratory of Theoretical Biology, National Cancer Institute,
National Institutes of Health, Bethesda, Maryland 20205

Many applications envisioned for liposomes in cell biology and chemotherapy require their direction to specific cellular targets^{1–3}. The ability to use antibody as a means of conferring specificity to liposomes would markedly increase their usefulness. We report here a method for covalently coupling soluble proteins, including monoclonal antibody and *Staphylococcus aureus* protein A (ref. 4), to small sonicated liposomes, by using the heterobifunctional cross-linking reagent *N*-hydroxysuccinimide 3-(2-pyridylthio)propionate (SPDP, Pharmacia). Liposomes bearing covalently coupled mouse monoclonal antibody against human β_2 -microglobulin [antibody B1.1G6 (IgG2a, κ) (B. Malissen *et al.*, in preparation)] bound specifically to human, but not to mouse cells. Liposomes bearing protein A became bound to human cells previously incubated with the B1.1G6 antibody, but not to cells incubated without antibody. The coupling method results in efficient binding of protein to the liposomes without aggregation and without denaturation of the coupled ligand; at least 60% of liposomes bound functional protein. Further, liposomes did not leak encapsulated carboxyfluorescein (CF) as a consequence of the reaction.

Reaction of SPDP with molecules containing primary amino groups results in the introduction of dithiopyridyl groups through the formation of amide bonds and release of *N*-hydroxysuccinimide⁵. Protein-bound dithiopyridine (DTP) can be readily activated as the thiol derivative by reduction with

Table 1 Precipitation of protein-bearing liposomes by *S. aureus* strain Cowan 1 (SaCI)

Determination	¹²⁵ I-labelled protein on liposomes: B1.1G6	Protein A
¹²⁵ I-labelled protein	66%	3%
CF in liposomes	62%	–10%

¹²⁵I-labelled protein-bearing liposomes (20 μ l, from fraction 8 of the Sepharose 4B column chromatography) were mixed with 1% bovine serum albumin in buffered saline in duplicate 900- μ l volumes. To some tubes 100 μ l of 10% SaCI was added; to control tubes only medium was added. After 30 min at 4 °C, tubes were spun 15 min at 2,300g to precipitate the SaCI and bound liposomes. Values represent the % liposomes bound to SaCI, as measured by ¹²⁵I counts in the bacterial pellet, and by release of fluorophore by addition of Triton.

dithiothreitol in mild conditions (acidic pH). These thiolated proteins can be reacted with other DTP-substituted molecules to produce covalently coupled products in conditions where homopolymerization and intramolecular cross-linking are kept to a minimum. For coupling of proteins to liposomes we have reacted SPDP with phosphatidylethanolamine (PE) in organic solvent, to form the stable derivative PE-DTP. This compound can be mixed with phosphatidylcholine and cholesterol, and liposomes formed by sonication. Protein containing activated thiol groups can then be coupled.

Figure 1 shows fractions eluted from a small Sepharose 4B column following coupling reactions between liposomes containing 4% PE-DTP and thiolated B1.1G6 (Fig. 1a, b), or thiolated protein A (Fig. 1c, d). Counts of ¹²⁵I-labelled protein are seen in the void volume for both proteins (Fig. 1a, c) only when liposomes were present. Figure 1 also shows control incubations of ¹²⁵I-labelled proteins incubated alone. In reactions carried out with ¹⁴C-labelled lipid and unlabelled protein (Fig. 1b, d) it can be seen that the liposome peak corresponds to the position of elution of protein in the void volume. This peak is also shown by fluorescence. Due to the high initial concentration of CF (40 mM), the encapsulated CF is strongly quenched^{6–8}, but the quenching can be relieved by the addition of detergent. The marked increase of fluorescence seen in fractions 7–9 (Fig. 1b, d) after addition of Triton X-100 confirms the position of the liposome peak. The same panels indicate that there has been only a slight leakage of liposome contents—that is, the fluorescence not increased by detergent (fractions 15–17)—during the

Table 2 Binding of fluoresceinated proteins or CF-containing protein-bearing liposomes to 5×10^6 human peripheral blood leukocytes

Fluorescent reagent	pmol protein incubated	pmol CF or fluorescein		
		Incubated	Bound	
			+Antibody	–Antibody
FITC-protein A (FPA)	30	120	12	1.4
Protein A liposomes	3.2	118	19	1.0
FITC-goat F(ab') ₂				
anti-mouse Ig (F-GaM)	110	165	7.7	0.2
B1.1G6 liposomes	1.3	103	ND	23*

Human peripheral blood leukocytes obtained by standard techniques were incubated in tissue culture medium containing 5% fetal calf serum, in the presence of 25 μ g ml^{–1} B1.1G6 or without antibody. After washing, the cells were mixed with the indicated quantities of protein-bearing liposomes (from fraction 8 of Sepharose 4B column chromatography, Fig. 1), with FPA (Pharmacia) or with F-GaM (Cappel). All incubations were at 4 °C in the presence of 0.02% Na₂S₂O₃. After washing, the cell pellets were lysed with 0.5% Triton and the supernatant measured for fluorescence. The fluorescence of fluoresceinated proteins is corrected for quenching, which is not relieved by Triton. The degree of quenching was determined by the difference between the fluorescence observed and that expected (excitation, 488 nm; emission, 520 nm) for the fluoresceinated proteins for the quantity of fluorescein found to be present by absorption (*A*₄₈₈), with respect to an unquenched standard. Fluorescein in FPA is 78% quenched and in F-GaM is 44% quenched. The CF in liposomes could be measured directly by fluorescence after relief of quenching by addition of Triton; it was 84–86% quenched in intact liposomes. The quantities of fluorescein or CF incubated and bound were determined by comparison with a known concentration of CF, which has the same fluorescence quantum efficiency as fluorescein. ND, not determined.

* Liposomes incubated with 5×10^6 mouse spleen cells bound 2.8 pmol CF.

reaction. We have not observed ^{125}I -labelled protein in the void volume when we chromatographed liposomes and proteins containing DTP groups but not activated by reduction (our unpublished data).

Table 1 shows the results of a representative experiment demonstrating that the protein excluded in the void volume is, in fact, coupled to the surface of the liposomes. This experiment takes advantage of the fact that cells of *S. aureus*, strain Cowan I (SaCI), which express protein A on their surface, will bind to and precipitate certain classes of antibody⁹, including the B1.1G6 class. The result demonstrates that the binding of B1.1G6 antibody to liposomes does not impair recognition of the Fc region of the antibody by the SaCI. Liposomes bearing the covalently bound soluble homologous protein A were not precipitated by the SaCI. The co-precipitation of CF with ^{125}I -

labelled B1.1G6 indicated that at least 60% of liposomes bear on their surface antibody molecules whose affinity for SaCI is unimpaired.

Liposomal B1.1G6 is also functional with respect to its antibody-combining site (Table 2). B1.1G6-bearing liposomes became bound to human cells, but not to mouse cells. Human cells preincubated with the B1.1G6 antibody and then washed, bound protein A-bearing liposomes; few liposomes were bound if cells were not incubated with the antibody. In experiments not presented here mouse cells preincubated with the monoclonal anti-I-A^k antibody 10-2-16 (ref. 10) also bound protein A liposomes. The use of protein A on liposomes would thus permit aliquots of a single liposome preparation to be directed at multiple targets, limited only by the availability of antibodies of the appropriate specificity and class.

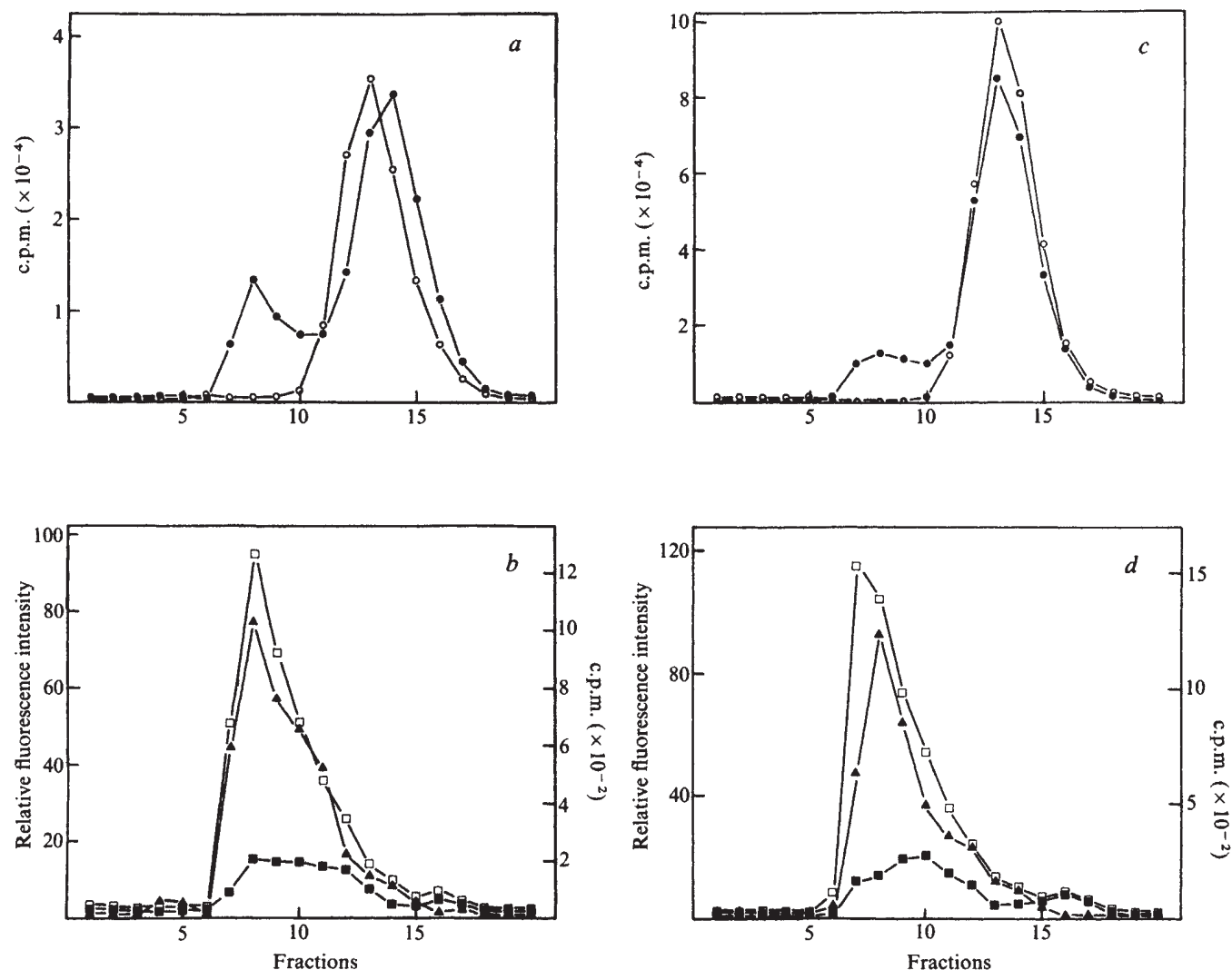


Fig. 1 Column chromatography and preparation of protein-bearing liposomes. Protein bound to liposomes or proteins alone were fractionated as follows. The liposome-protein reaction mixture (400 μl) or 200 μl of ^{125}I -labelled reduced proteins were applied to a 1.5×5 cm column of Sepharose 4B run at 7.2 ml h^{-1} ; fractions are 0.6 ml. Aliquots were measured for ^{125}I , ^{14}C and also for CF fluorescence. Fluorescence measurements were made with a Farrand Mark I spectrofluorometer (excitation 488 nm; emission 520 nm) before and after releasing entrapped CF with 0.5% Triton X-100. An unquenched fluorescence standard (66 nM CF) was set at unity for the scale. *a, b*, B1.1G6 and liposomes; *c, d*, protein A and liposomes. The liposome-protein coupling reaction was performed as follows. Dipalmitoyl phosphatidylethanolamine (DPPE, Calbiochem), 10 μmol , was mixed in chloroform/methanol 9:1 (C:M) with ^{14}C -PE (NEN) to a final specific activity of $0.15 \mu\text{Ci } \mu\text{mol}^{-1}$. To 10 μmol was added 12 μmol SPDP and 20 μmol triethylamine. After 2 h at 25°C the organic phase was extracted with water, dried and the product redissolved in C:M to a final concentration of $5 \mu\text{mol ml}^{-1}$. TLC on silical gel revealed a single spot with R_f 0.47 (50 μg sample in $\text{CHCl}_3/\text{MeOH}/\text{H}_2\text{O}$ 65:25:4). The yield (DPPE-DTP) was 90%, based on ^{14}C . For the formation of liposomes: to 10 μmol dipalmitoyl phosphatidylcholine (DPPC, Sigma) was added 5 μmol cholesterol (Applied Science Labs) and 0.6 μmol ^{14}C -DPPE-DTP, all in C:M. Solvent was evaporated and liposomes formed by sonication under N_2 for 40 min at 45°C in the presence of 3 ml 40 mM CF in 0.01 M HEPES-buffered saline, pH 7.45. Unencapsulated CF was removed by gel filtration. ^{125}I -labelled or unlabelled proteins, 2.4 mg ml^{-1} , were incubated with 0.08 mM (for B1.1G6) or 0.5 mM (for protein A) SPDP at pH 7.45 for 30 min at 25°C . Unreacted SPDP was removed by gel filtration and protein-bound DTP was reduced with 50 mM dithiothreitol in 0.1 M acetate-buffered saline, pH 4.5, for 20 min. Liposomes (500 μl), at a concentration of 2 mM (in DPPC), were incubated overnight at 25°C with an equal volume of ^{125}I -labelled or unlabelled reduced protein, 1 mg ml^{-1} , pH 7.45. Samples for ^{14}C counting were incubated with unlabelled protein. $\bullet$, ^{125}I -labelled protein incubated with liposomes (*a, c*); $\circ$, ^{125}I -labelled protein alone (*a, c*); $\blacktriangle$, ^{14}C -PE in liposomes (*b, d*); $\blacksquare$, CF fluorescence before, and $\square$, after, addition of Triton (*b, d*).

Table 2 indicates another feature of liposome encapsulation, namely, the large quantity of material that can be carried. The fluorescein/protein (F/P) ratio of directly fluoresceinated antibody (using fluorescein isothiocyanate (FITC)) is limited by denaturation of the antibody to usually not more than 3 or 4 (ref. 11). (Protein A can have a somewhat higher F/P ratio without denaturation.) In this experiment, the F/P ratio for antibody-bearing liposomes is 50 times greater than that of the directly fluoresceinated antibody, and need only be limited by the solubility of the encapsulated material, which for CF is at least sixfold higher than that used here.

Because the coupling conditions minimize homopolymerization of proteins or liposomes, this technique avoids the aggregation and denaturation of IgG observed in techniques associating antibody and liposomes noncovalently, by sonication^{12,13}. We have introduced approximately two DTP groups per antibody molecule. In these conditions the titre of the protein is only minimally reduced in a sensitive radioimmunoassay (manuscript in preparation).

We have been unable to associate protein A with liposomes using the glutaraldehyde technique described by Torchilin *et al.*^{14,15}. Also, a technique has recently been reported¹⁶ for the formation of PE-derivatives of proteins by reacting them with an activated PE-derivative, following exposure of their intrinsic sulphhydryl residues. The protein-PE complex is then used for passive sensitization of liposomes. This method could not be used with protein A, as it lacks cysteine¹⁷. In addition, by analogy with 'active' against 'passive' incorporation of dinitrophenyl-PE into liposomes¹⁸, the inclusion of a prosthetic group into liposomes at the time they are formed, as in the present case, is likely to be a more efficient process.

In experiments to be presented elsewhere, we have encapsulated methotrexate in liposomes to which we have covalently coupled antibody and protein A. In this context we are extending our studies on immunological targeting of methotrexate in liposomes^{19,20}. We have also used SPDP to couple albumin, avidin and affinity-purified rabbit antibody to liposomes, and the technique can, in principle, be applied to any compound containing an available amino group. Thus, encapsulation and specific targeting of numerous water-soluble compounds of biological and chemotherapeutic interest is quite feasible.

Pierre Henkart suggested the use of SaCl to precipitate antibody-bearing liposomes. This research was supported by funds from INSERM, CNRS and DRGST (ACC FK, 1980).

Note added in proof: A detergent dialysis technique for the association of monoclonal antibodies with liposomes has recently been reported²¹.

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- Gregoriadis, G. (ed.) *Drug Carriers in Biology and Medicine* (Academic, London, 1979).
- Gregoriadis, G. & Allison, A. C. (eds) *Liposomes in Biological Systems* (Wiley, New York, 1980).
- Tom, B. H. & Six, H. R. (eds) *Liposomes and Immunobiology* (Elsevier, Amsterdam, in the press).
- Goding, J. W. *J. Immun. Meth.* **20**, 241-253 (1978).
- Carlsson, J., Dreven, H. & Axén, R. *Biochem. J.* **173**, 723-737 (1978).
- Weinstein, J. N., Yoshikami, S., Henkart, P., Blumenthal, R. & Hagins, W. A. *Science* **195**, 489-492 (1977).
- Weinstein, J. N., Blumenthal, R., Sharrow, S. O. & Henkart, P. *Biochim. biophys. Acta* **509**, 289-299 (1978).
- Leserman, L. D., Weinstein, J. N., Blumenthal, R., Sharrow, S. O. & Terry, W. D. *J. Immun.* **122**, 585-591 (1979).
- Cullen, S. E. & Schwartz, B. D. *J. Immun.* **117**, 136-142 (1976).
- Oi, V. T., Jones, P. P., Goding, J. W., Herzenberg, L. A. & Herzenberg, L. A. *Curr. Topics Microbiol. Immun.* **81**, 115-129 (1978).
- Forni, L. in *Immunological Methods* (eds Lefkowitz, I. & Pernis, B.) 151-166 (Academic, London, 1979).
- Gregoriadis, G. & Neerunjun, D. E. *Biochem. biophys. Res. Commun.* **65**, 537-544 (1975).
- Huang, L. & Kennel, S. J. *Biochemistry* **18**, 1702-1707 (1979).
- Torchilin, V. P., Goldmacher, V. S. & Smirnov, V. N. *Biochem. biophys. Res. Commun.* **85**, 983-985 (1978).
- Torchilin, V. P., Khaw, B. A., Smirnov, V. N. & Haber, E. *Biochem. biophys. Res. Commun.* **89**, 1114-1119 (1979).
- Sinha, D. & Karush, F. *Biochem. biophys. Res. Commun.* **90**, 554-560 (1979).
- Sjodahl, J. *Eur. J. Biochem.* **78**, 471-490 (1977).
- Uemura, K. & Kinsky, S. C. *Biochemistry* **11**, 4085-4094 (1972).
- Leserman, L. D., Weinstein, J. N., Moore, J. J. & Terry, W. D. *Cancer Res.* (in the press).
- Leserman, L. D., Weinstein, J. N., Blumenthal, R. & Terry, W. D. *Proc. natn. Acad. Sci. U.S.A.* **77**, 4089-4093 (1980).
- Huang, A., Huang, L. & Kenney, S. J. *J. biol. Chem.* **255**, 8015-8018 (1980).

Cell cycle kinetics in human lymphocyte cultures

Kanehisa Morimoto* & Sheldon Wolff

Laboratory of Radiobiology and Department of Anatomy, University of California, San Francisco, San Francisco, California 94143

Short-term cultures of phytohaemagglutinin (PHA)-stimulated human lymphocytes are widely used to detect chromosome-damaging agents^{1,2}, possible human exposure to mutagenic carcinogens^{1,2} and the immune response of blood³. Because the results are affected by the number of cell divisions before sampling^{1,2,4,5}, an accurate knowledge of lymphocyte proliferation in culture is essential for these studies. Unfortunately, the information available on the lymphocyte proliferative characteristics is quite conflicting. For instance, although after stimulation of blood lymphocytes with PHA the cultures soon contain cells that have divided different numbers of times^{6,7}: this heterogeneity has been explained variously as a difference in cell-cycle times^{8,9} or in the times when the cells start blastogenesis by responding to PHA¹⁰⁻¹². Prolonged treatment with high concentrations of ³H-thymidine (TdR) have often been used to investigate lymphocyte proliferation^{8,10,13,14}. Incorporated ³H-TdR can, however, affect cell kinetics¹⁵⁻¹⁹. The differential staining of sister chromatids²⁰ in cells dividing for different numbers of times in the presence of bromodeoxyuridine (BUdR) can be used to study cell kinetics^{7,8,12}. In experiments combining sister chromatid differential staining and autoradiography, we show here that ³H-TdR labelling at more than 0.1 μ Ci ml⁻¹ slows lymphocyte cycling, and that the heterogeneity of different generations of cells is caused by a difference in the times when they start their first DNA synthesis in response to PHA.

PHA-stimulated human lymphocytes were cultured in medium containing BUdR so that cells dividing for the first, second and third time after initiation of culture could be distinguished by the differential staining pattern of sister chromatids in slides stained with fluorescence-plus-Giemsa (FPG)²⁰. When metaphase cells from cultures were examined at various times after initiation, it was found that the relative numbers of 1st, 2nd and 3rd or subsequent generation cells shift with increasing culture times (data not shown, see ref. 7). We have already shown that cell division delay induced by chemical treatments is clearly manifested as shifts in the relative proportion of these metaphases⁷.

To measure the effect of ³H-TdR on cell proliferation, a simple combination method of sister chromatid differential staining and autoradiography was developed (Fig. 1). Cultures were established with 20 μ M BUdR and exposed from the 32nd to the 40th hour to ³H-TdR (specific activity, 52 Ci mmol⁻¹) at concentrations of 0.05-5 μ Ci ml⁻¹. Consequently, at the highest concentration used the medium contained only 0.1 μ M thymidine. An 8-h treatment was selected, because treatments for 4-24 h are commonly used for labelling human lymphocytes in culture. The cultures were then fixed at 64 h and the numbers of cells dividing for the first, second or third time determined in FPG-stained slides^{7,12}.

At concentrations above 0.2 μ Ci ml⁻¹, ³H-TdR affects the distribution of each generation metaphase cells in a clear dose-dependent manner (Fig. 2a). In 64-h cultures exposed to ≤ 0.2 μ Ci ml⁻¹ ³H-TdR, approximately 10% of the metaphases were in their first division, 40% in their second division and 50% in their third. In cultures exposed to more than 0.2 μ Ci ml⁻¹ ³H-TdR, the proportion of 1st-generation metaphases increased, whereas that of 3rd-generation metaphases

* Permanent address: Department of Public Health, Faculty of Medicine, University of Tokyo, Tokyo 113, Japan.

Table 1 Incidence of labelled metaphases in 64-h cultures pulse-treated with ^3H -TdR

Labelling period (h after initiation)	Donor	% Labelled metaphases	% Labelled (or heavily labelled) metaphases among:			
			X ₁ I	X ₂ I	I	X ₃ II
24-32	A	53.0	0 (0)	2.9 (0)	96.3 (63.0)	0 (0)
	B	43.0	0 (0)	10.6 (2.1)	97.4 (72.0)	0 (0)
32-40	A	49.0	0 (0)	89.2 (37.8)	29.2 (6.3)	4.2 (0)
	B	53.0	0 (0)	90.6 (52.6)	35.6 (5.1)	7.0 (1.8)
40-48	A	85.0	25.0 (11.8)	85.3 (41.2)	0 (0)	98.1 (79.6)
	B	65.0	31.6 (15.8)	59.2 (32.7)	0 (0)	93.8 (78.1)

Labelling was with $0.1 \mu\text{Ci ml}^{-1}$ ^3H -TdR. I or II represents the metaphases whose labelling pattern indicates the ^3H -TdR incorporation in the first or second cell cycle, respectively (for details see Fig. 1 legend). The criteria for labelling and the abbreviations used were the same as those in Fig. 2. For metaphases whose labelling pattern indicated ^3H -TdR incorporation in the second S phase (Fig. 1), if more than one-third (or two-thirds) of the chromosomes were labelled on both chromatids (for 2nd-generation metaphases) or on lightly stained chromatids (for 3rd-generation metaphases), they were designated as labelled (or heavily labelled).

decreased. The percentage of 2nd-generation metaphases exhibited a small peak at $0.5 \mu\text{Ci ml}^{-1}$ and then at higher concentrations decreased more markedly than did the 3rd-generation metaphases, apparently indicating that the induced mitotic delay in 2nd-generation cells might be longer than that in 3rd-generation cells.

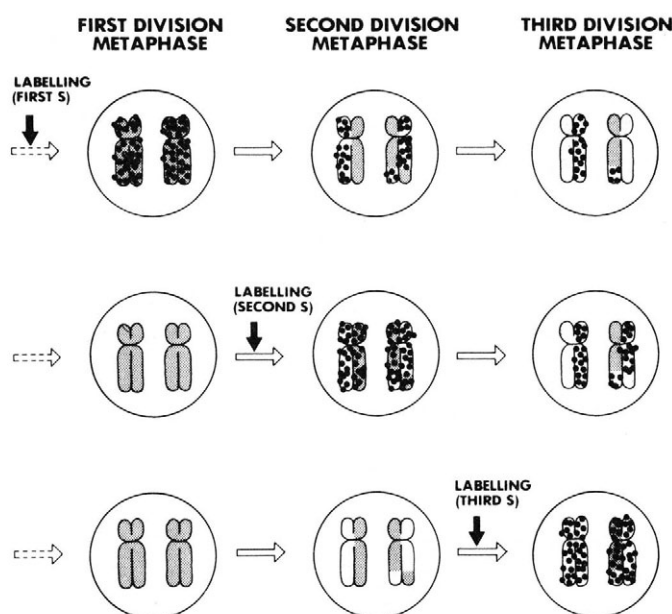


Fig. 1 Schematic presentation of the simple combination method of ^3H -TdR labelling and sister chromatid differential staining. When cells are incubated continuously with BUdR and pulse-labelled with ^3H -TdR at different times after initiation of culture, metaphase cells from resultant cultures have various combinations of the differential staining pattern of sister chromatids and the distribution of autoradiographic silver grains over the chromosomes. The differential staining pattern of sister chromatids yields information on how many times a cell has divided in cultures, and the distribution of autoradiographic grains over the chromosomes indicates when the cell synthesized DNA. 1st-Division cells contain chromosomes with both sister chromatids stained uniformly darkly. 2nd-Division cells contain only harlequin chromosomes with one chromatid darkly stained and its sister chromatid lightly stained, whereas 3rd-Division cells contain some harlequin chromosomes and other chromosomes with both sister chromatids stained uniformly lightly. For cells which have incorporated ^3H -TdR in their first S phase, a 1st-division cell has labelling on both chromatids of a chromosome whereas a 2nd-division cell is labelled only on lightly stained chromatids. A 3rd-division cell has labelling on lightly stained chromatids only where both chromatids of a chromosome are lightly stained. For cells which have incorporated ^3H -TdR in their second S phase, a 1st-division cell has no labelling at all, a 2nd-division cell has labelling on both chromatids and a 3rd-division cell is labelled on lightly stained chromatids where both chromatids of a chromosome are lightly stained as well as where one is lightly stained and one is darkly stained. Such combination patterns of labelling and sister chromatid differential staining are the same in whichever division cycle sister chromatid exchanges take place, although here only those which occurred in the 2nd cell cycle are shown for clarity.

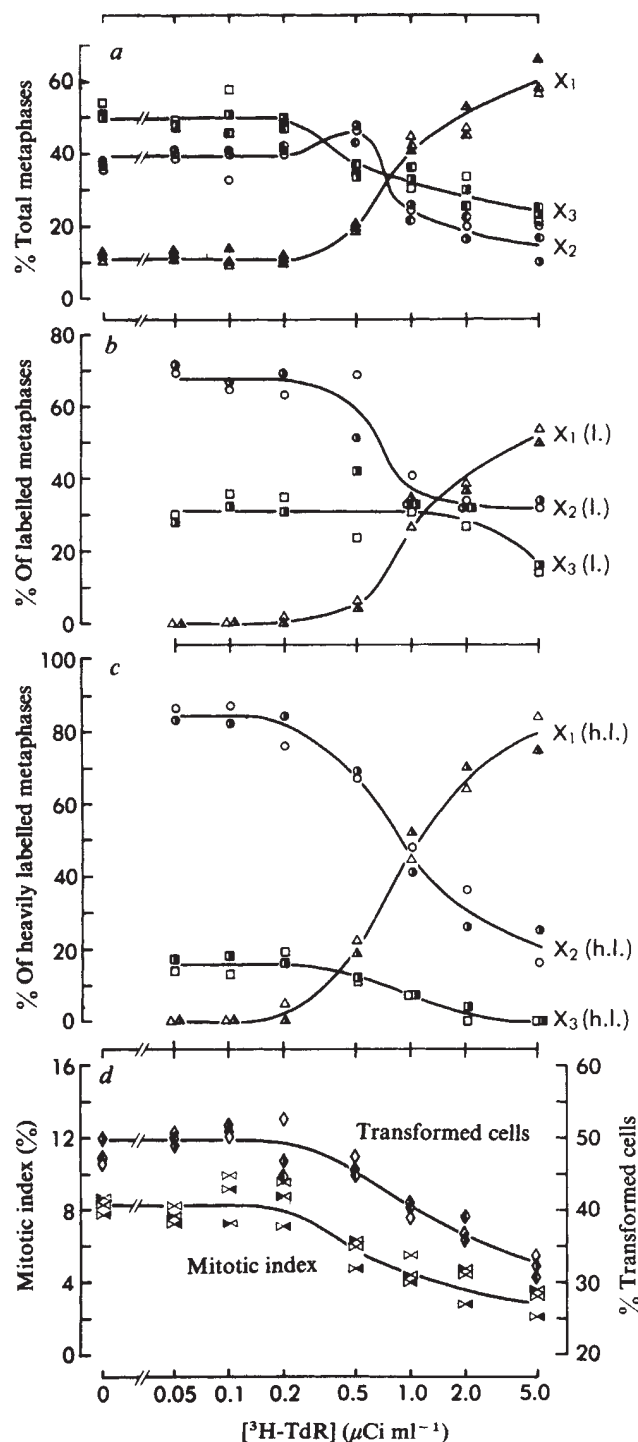
Autoradiograms of the same slides showed that the shift in the relative proportions was more pronounced among labelled metaphases (Fig. 2b), being much more marked in heavily labelled metaphases (Fig. 2c). 'Unlabelled' metaphases, categorized as those other than the labelled cells, showed a small shift in the relative proportions when exposed to more than $1 \mu\text{Ci ml}^{-1}$ ^3H -TdR (data not shown). The data also indicated that, with exposure to ^3H -TdR between 32 and 40 h of culture, many more 2nd- than 3rd-generation cells had been labelled in their first S phase (Fig. 2b, c) and that the apparently longer mitotic delay in 2nd-generation metaphase cells (Fig. 2a) was only a reflection of such differential labelling. Labelled 1st-generation metaphases were found only in the cultures exposed to more than $0.1 \mu\text{Ci ml}^{-1}$ ^3H -TdR. Corresponding decreases in both the mitotic indices and the percentage of transformed cells (Fig. 2d) also occurred. These data indicate that ^3H -TdR labelling with concentrations above $0.1 \mu\text{Ci ml}^{-1}$ slows lymphocyte proliferation in culture.

In preliminary experiments we have noted that cell division kinetics are not perturbed by washing the cells and replacing the medium, by treatment with as high as $10 \mu\text{M}$ cold thymidine, or by the use of BUdR at concentrations up to $40 \mu\text{M}$ (data not shown). We thus believe that the observed mitotic delay is due to incorporated ^3H -TdR.

Autoradiograms of FPG-stained cells labelled with $0.1 \mu\text{Ci ml}^{-1}$ ^3H -TdR at different times after initiation of culture have also shown (Table 1) that the majority of 2nd-generation metaphases seen in a 64-h culture started DNA synthesis at 32-40 h after initiation, and that none of the 1st-generation metaphases started DNA synthesis before 40 h, a quarter of them entering their first S phase at 40-48 h. Most of the 3rd-generation metaphases entered their first S phase at 24-32 h and their second S phase at 40-48 h. These data thus indicate that in cultures, the older-generation cells begin their first DNA synthesis at earlier times after the start of incubation. As shown in Fig. 2, treatment with higher concentrations of ^3H -TdR perturbs cell division kinetics such that cells with more incorporated ^3H -TdR have longer cell-cycle times.

Crossen and Morgan⁸ have recently found that the small proportion of 1st-division metaphases seen in 72-h cultures treated with $2 \mu\text{Ci ml}^{-1}$ ^3H -TdR entered S phase before the 34th hour of incubation. We think that this observation of extraordinarily long cell-cycle times is the result of prolonged G_2 phases caused by the radiation from incorporated ^3H -TdR.

Pollack *et al.*¹⁹ also showed by flow cytometric (FCM) analysis that an 18-h treatment with $0.5 \mu\text{Ci ml}^{-1}$ ^3H -TdR increased the relative proportion of lymphocytes in the G_2 plus M phase, suggesting an inhibition of cell proliferation. Such a G_2 block, measured by FCM analysis, was also reported in several mammalian cell lines treated with low concentrations of ^3H -TdR^{17,18}. However, the elevation in the G_2 + M peak does not always indicate an actual delay in cell proliferation because the FCM analysis provides only a static representation of the distribution of cells about the cell-cycle phases¹⁷⁻¹⁹ and because a transient



increase of cells in the $G_2 + M$ can be observed with an unaltered population doubling time of cultured cells^{17,18}. Here we have shown directly that an 8-h treatment with as low as $0.2 \mu\text{Ci ml}^{-1}$ ^3H -TdR delays lymphocyte proliferation in culture.

Purrott *et al.*²¹ showed by FPG-differential staining of PHA-stimulated lymphocytes that X-ray irradiation of G_0 cells induces a mitotic delay of 1 h Gy^{-1} . In the present experiments accurate estimations of the dose from incorporated ^3H -TdR and of the amount of delay are not possible because of the complications of both the uptake of ^3H -TdR and the rate of cell division. Assuming a dose of approximately 5 mGy per disintegration²², however, the dose rate received by labelled cells in the first cell cycle after a treatment of $0.5 \mu\text{Ci ml}^{-1}$ ^3H -TdR can be estimated to be roughly 30 mGy h^{-1} . The mitotic delay observed with this treatment is 4–6 h, which was estimated from

Fig. 2 ^3H -TdR-induced changes in the relative proportion of metaphase cells in the 1st (X₁, triangles), 2nd (X₂, circles) or 3rd (X₃, squares) division in 64-h cultures. Changes in the proportion among (a) total, (b) labelled (l.), or (c) heavily labelled (h.l.) metaphases are shown, as are (d) changes in the mitotic indices and the percentage of transformed cells. Two healthy human males aged 30 yr (open symbols) and 33 yr (half-closed symbols) were used as blood donors. Cells were treated with ^3H -TdR at the appropriate concentration from the 32nd to the 40th hour after initiation of cultures. The treatment with ^3H -TdR was terminated by washing the cells three times and resuspending them in prewarmed complete medium. A 64-h incubation time was selected because preliminary experiments indicated that the cultures would contain relative proportions of 1st-, 2nd- and 3rd-division metaphases appropriate for the observation of induced perturbations in cell division kinetics. In FPG-stained preparations, cells dividing for the first, second or third time in culture were determined by the differential staining pattern of sister chromatids (Fig. 1). This experiment was repeated with blood from the same donor (left-half-closed symbols). For autoradiography of FPG-stained cells, slides were dipped in a 1% Formvar solution²³ to prevent the formation of chemical grains by direct contact between the autoradiographic nuclear emulsion and the Giemsa stain. The slides were then dipped in Ilford L-4 emulsion and developed after 1–90 days exposure at 4°C . The exposure time was inversely proportional to the ^3H -TdR concentrations. The degree of labelling was determined by the number of chromosomes labelled in a cell. A 1st-division metaphase was designated as labelled if more than one-third of the chromosomes were labelled on both chromatids; if more than two-thirds of the chromosomes were so labelled the cell was considered to be heavily labelled. Similarly, a second division cell was considered labelled (or heavily labelled) if more than one-third (or two-thirds) of the chromosomes were labelled on the light chromatid. 3rd-Division cells were considered labelled (or heavily labelled) if labelling occurred in the lightly stained chromatids of more than one-sixth (or one-third) of those chromosomes with both chromatids lightly stained in the centromeric regions. These criteria were applied for labelled metaphase cells that incorporated ^3H -TdR in their first S phase after initiation. Metaphase cells whose labelling pattern indicated ^3H -TdR incorporation in the second S appeared more frequently in cultures treated at the higher concentrations of ^3H -TdR. These cells can also be used to indicate cell division delay (data not shown). From each culture 200 metaphases were scored for the distribution of 1st-, 2nd- and 3rd-division cells, and 100 metaphases from each culture were analysed for the distribution of labelled metaphases. Mitotic indices and the percentage of transformed cells were based on 2,000 cells.

a dose-dependent change in the distribution of 1st-, 2nd- and 3rd-division cells in ^3H -TdR-treated and control cultures⁷.

The present study has thus shown the simple combination of autoradiography and differential staining of sister chromatids to be a sensitive method for detecting mitotic delay in human lymphocyte cultures. It can be easily applied to other cell cultures to check the effect of ^3H -TdR labelling on cell proliferation. Our data suggest that some information available on human lymphocyte proliferation might be misleading because they were often obtained with lengthy ^3H -TdR labelling at high concentrations, which induce mitotic delay. We have also presented direct evidence that the heterogeneity with respect to different generation metaphases seen in a culture is a reflection of a difference in the times the cells enter their first DNA-synthesis period, and that the cells have about the same generation times thereafter.

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- Evans, H. J. & Lloyd, D. C. *Mutagen-Induced Chromosome Damage in Man* (Yale University Press, New Haven, 1978).
- Evans, H. J., Court Brown, W. M. & McLean, A. S. *Human Radiation Cytogenetics* (North-Holland, Amsterdam, 1967).
- Oppenheim, J. J. & Rosenstreich, D. L. *Prog. Allergy* **20**, 65–194 (1976).
- Bender, M. A. & Brewen, J. G. *Mutat. Res.* **8**, 383–399 (1969).
- Savage, J. R. K. *Nature* **278**, 687 (1979).
- Tice, R., Schneider, E. L. & Rary, J. M. *Expl Cell Res.* **102**, 232–236 (1976).
- Morimoto, K. & Wolff, S. *Cancer Res.* **40**, 1189–1193 (1980).
- Crossen, P. E. & Morgan, W. F. *Expl Cell Res.* **118**, 423–427 (1979).
- Craig-Holmes, A. P. & Shaw, M. W. *Expl Cell Res.* **99**, 79–87 (1976).
- Soren, L. *Expl Cell Res.* **78**, 201–208 (1973).
- Younkin, L. H. *Expl Cell Res.* **90**, 374–380 (1975).
- Tice, R., Thorne, P. & Schneider, E. L. *Cell Tissue Kinet.* **12**, 1–9 (1979).
- Sasaki, M. S. & Norman, A. *Nature* **210**, 913–914 (1966).
- Beek, B. & Obe, G. *Hum. Genet.* **35**, 57–70 (1976).
- Drew, R. M. & Painter, R. B. *Radiat. Res.* **11**, 535–544 (1959).
- Cleaver, J. E. *Thymidine Metabolism and Cell Kinetics* (North-Holland, Amsterdam, 1967).
- Ehmann, U. K. *et al. Nature* **258**, 633–636 (1975).
- Marz, R. *et al. J. cell. Physiol.* **90**, 1–8 (1977).
- Pollack, A., Bagwell, C. B. & Irvin, G. L. *III Science* **203**, 1025–1027 (1979).
- Perry, P. & Wolff, S. *Nature* **251**, 156–158 (1974).
- Purrott, R. J., Vulpis, N. & Lloyd, D. C. *Mutat. Res.* **69**, 275–282 (1980).
- Cleaver, J. E., Thomas, G. H. & Burki, H. J. *Science* **177**, 996–998 (1972).
- Rutledge, M. H. *Chromosoma* **70**, 259–262 (1979).

Effects of an Earth-strength magnetic field on electrical activity of pineal cells

P. Semm, T. Schneider & L. Vollrath

University of Mainz, Department of Anatomy, Neurophysiology Laboratory, 6500 Mainz, Saarstr. 19/21, FRG

Although magnetic fields can influence biological systems, including those of man and other vertebrates¹⁻⁵, no central nervous structure has been identified that might be involved in their detection. From a theoretical point of view, the pineal organ might be such a structure for the following reasons: (1) It is involved in the regulation of circadian rhythms⁶ and is thus essential for migratory restlessness ('Zugunruhe')⁷. Orientation at that time can be altered by an artificial magnetic field (MF) with a direction differing by 90° from that of the Earth. Circadian rhythms can be inhibited from phase shifting by compensation of the Earth's MF and can be influenced by an artificial MF⁸. (2) The pineal organ is strongly dependent on its sympathetic innervation⁶ and the sympatho-adrenergic system as a whole is sensitive to magnetic stimuli⁹. (3) The pineal organ is a light-sensitive time-keeping organ^{10,11} and could form part of a combined compass-solar-clock system, which has been postulated for maintaining orientation in birds¹². We have therefore investigated the effect of a MF on electrophysiological activity of the guinea pig pineal organ, which is a useful system for such studies on individual cells^{11,13}. We report here that activity was depressed by an induced MF and restored when the MF was inverted.

We abandoned attempts to use a small permanent magnet, as the MF produced was neither homogeneous nor accurately measurable in the area of the pineal organ. We used 16 male guinea pigs (300–900 g), anaesthetized with urethane. In each experiment a tracheotomy was performed, and the animal artificially respiration and mounted in a stereotaxic frame. Body temperature was maintained using a heating pad and the electrocardiogram continuously monitored. The dorsal surface of the brain was exposed, and a single micropipette inserted into the posterior part of the pineal organ, just under the confluence of the sagittal and transverse sinus. Extracellular activity was

recorded through the micropipette, filled with 4 M NaCl solution saturated with fast green for verifying recording sites histologically. The MF was generated by two Helmholtz coils (radius 3.6 cm), one under the jaw and the other on top of the head, with 3.6 cm distance between them. The strength of the MF in the middle of the two coils was

$$B_H = \left(\frac{4}{5}\right)^{\frac{3}{2}} \cdot \frac{\mu_0 \cdot n \cdot I}{R}$$

where B_H is the magnetic field strength, μ_0 the permeability of free space, R the radius (3.6 cm), n the winding number (150) and I the intensity of current (for example: I_1 for 0.3 Oe = 8.0073 mA and I_2 for 0.8 Oe = 21.3528 mA). The MF thus calculated was measured by a Hall-sound and was shown to be relatively homogeneous around the animal's head. The direction of the current and thereby the polarity of the MF could be altered. The artificial MF was added to the vertical component of the Earth's MF when the top coil was made the south pole. It compensated, or inverted, the vertical component when the top coil was made the north pole. The activity maintained by the cells under study was averaged by an averager system with a time base of 1 s per bin (total bin number = 256; a bin is the period of time during which the incoming electrical potentials are summed). The spike number of each averager histogram was determined by an electric counter. The histograms were read and plotted on a recorder every 4 min 16 s.

Before the MF stimulus was applied, we checked that short-term optic and acoustic stimuli had no effect on the pineal cells and that the maintained activity of a given cell was relatively constant (measured for about 16 min, Fig. 1b, c). Then the first MF stimulus of 0.5 Oe was applied for 17 min 4 s and, after 8 min, a second stimulus of the same strength for 8 min. Of a total of 71 cells tested in 16 male guinea pigs, 56 did not respond (Fig. 1a). A cell was classified as sensitive to stimulation only when spontaneous electrical activity was depressed by more than 30%. Fifteen cells (from 11 animals) showed a marked response to MF stimulation; as Fig. 1b and c show, maintained activity was diminished by more than 50%, well out of the range of spontaneous oscillations of pineal electrical activity. The first clear effect could be measured after a latency period of about 2 min, not visible in the figure due to averaging at 4 min 16 s intervals. When the MF was switched off, all cells retained a diminished activity, lasting as long as recording could be continued (up to 30 min in some cases). A second round of the same stimulus had no effect (Fig. 1b, c) even when the strength of the stimulus was varied (not shown).

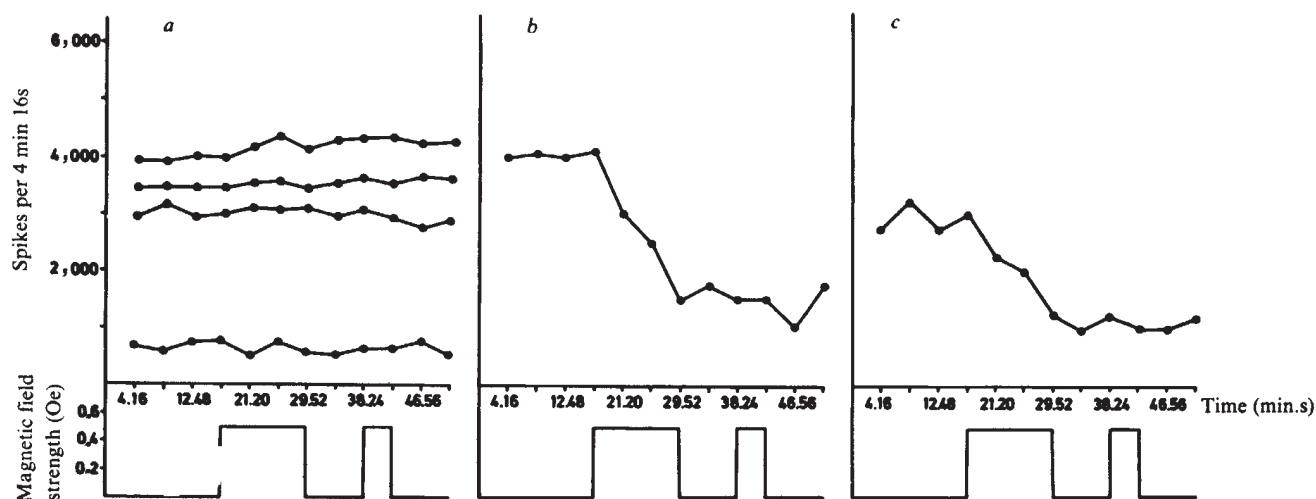


Fig. 1 a, The number of spikes from four single pineal cells (counted every 4 min 16 s, averaging time) which could not be influenced by a magnetic stimulus of 0.5 Oe. b, Reaction of a pineal cell to a MF of 0.5 Oe. c, Another pineal cell, the maintained activity of which is diminished by a MF of 0.5 Oe. A second stimulus had no effect in either cell.

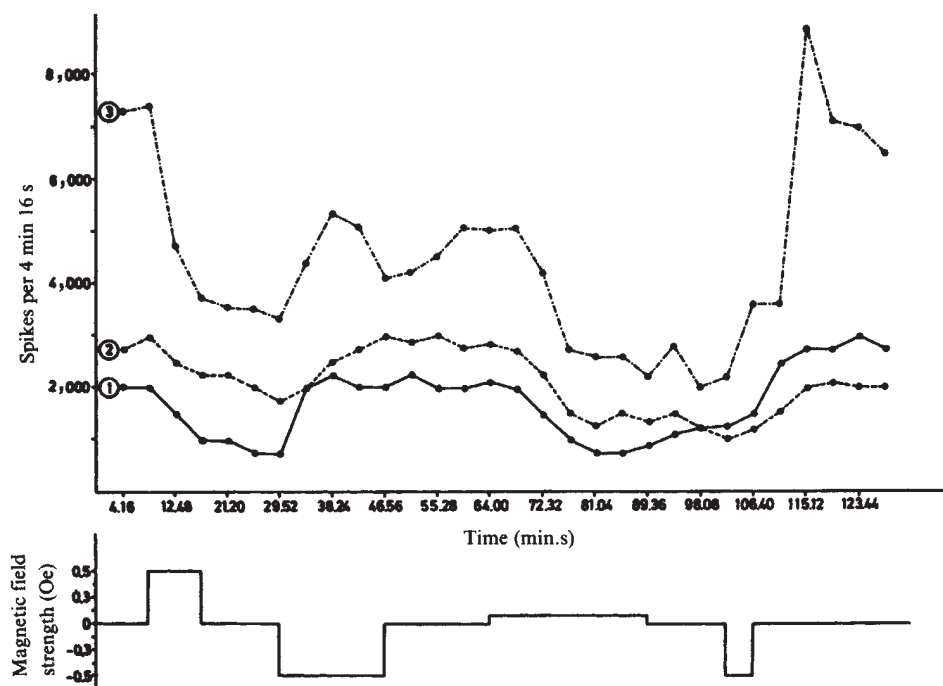


Fig. 2 The spike number of three single pineal cells. Cell no. 1 shows a clear depression of activity after a magnetic stimulus of 0.5 Oe and remains depressed after cessation of the stimulus. After an inverted stimulus of 0.5 Oe, cell activity returns to output level and is depressed again by a stimulus of 0.10 Oe, south pole up. After a period of depression, activity increases again after a short stimulus of 0.5 Oe with inverted polarity of MF. Cell no. 2 shows a similar reaction, whereas cell no. 3 does not reach its output level after the first application of the inverted MF. After the second inverted magnetic stimulus, cell activity reached output level again.

When the polarity of the MF was changed in 3 of the 15 responsive cells, their activity immediately returned to the initial level (Fig. 2). With the exception of cell no. 3, they all reached this level after 8–12 min. The latency of the renewed activation was also in the range of 2 min (not shown). When the inverted MF was switched off the cells retained their normal activity, which could be depressed again by a low MF stimulus of 0.10 Oe, pointing to a relatively high sensitivity of the cells. After a further inversion of polarity, the cells were again activated. In one case maintained activity could be directly stimulated by an inverted MF (north pole up).

In other brain structures (inferior and superior colliculi, corpus callosum and epithalamus) in identical experimental conditions, no reactions to magnetic stimuli could be measured. Thus, the cellular response of cells to magnetic stimuli is not universal and the reactions measured were not due to magnetic or electrical interference with the recording system.

The role of the pineal organ has been clarified in only a few species. In photoperiodic mammals it influences the hypothalamo-pituitary-gonadal axis and controls the atrophy of the gonads during winter by an increased output of the hormone melatonin¹⁴. In birds the pineal is involved in the regulation of locomotor activity¹⁵. In various species the pineal organ is readily influenced by external (light, noise, ambient temperature, olfactory stimuli) and internal (sex steroids, noradrenaline) factors⁶. The heterogeneity of the pineal cells has been demonstrated in the guinea pig although individual functions remain to be elucidated. This may help to explain why not all cells we recorded clearly responded to MF stimuli.

As this is apparently the first report that the pineal responds to changes in an artificial MF, we can offer no corroborative evidence for our findings. Indirect evidence is that migratory restlessness and locomotor activity, which have been clearly shown to be affected by the pineal, can be influenced by artificial or natural MFs. We also feel it is significant that for orientation, in addition to sensing the Earth's MF, time-keeping ability is required¹². As the pineal organ is a sort of time-keeping device¹⁰, it might be an ideal site for integration, particularly as birds do not simply switch between magnetic and Sun compasses¹².

Whether or not the pineal organ itself senses changes of MF is not known. The organ is heavily innervated by sympathetic

fibres¹⁶ and the sympathetic nervous system can be affected by magnetic stimuli, so that the effect may well be indirect. The lack of appreciable amounts of iron in the pineal organ^{17,18} makes it unlikely that the magnetite (magnetic iron oxide) required for transducing magnetic stimuli in bacteria¹⁹ is present.

Interestingly, it has been proposed²⁰ for migratory birds and homing pigeons that the MF is detected in the lateral eyes, although phylogenetically the pineal organ corresponds to the median eye of lower vertebrates⁶. The pineal organ of higher vertebrates is derived from the photoreceptive pineal organ of lower vertebrates²¹ and is connected to the lateral eyes by the nucleus suprachiasmaticus and the sympathetic nervous system²².

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Note added in proof: Recent experiments carried out in our laboratory indicate that pineal cells of homing pigeons, which according to other authors use the natural magnetic field for orientation, also respond clearly to artificial magnetic fields.

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1. Barnothy, M. F. (Plenum, New York, 1964).
2. Barnothy, M. F. Vol. 2 (Plenum, New York, 1969).
3. Markl, H. in *Biophysik* (eds Hoppe, W., Lohman, W., Markl, H. & Ziegler, H.) (Springer, Berlin, 1977).
4. Martin, H. & Lindauer, M. *Fortschr. Zool.* **21**, 211–228 (1973).
5. Martin, H. & Lindauer, M. *J. comp. Physiol.* **122**, 145–187 (1977).
6. Wurtman, R. J., Axelrod, J. & Kelly, D. E. *The Pineal* (Academic, New York, 1968).
7. McMillan, J. P. *J. comp. Physiol.* **79**, 105–112 (1972).
8. Brown, F. A. & Sciw, K. M. *J. interdisciplinary Cycle Res.* **9**, 137–145 (1978).
9. Sakharova, S. A. *Biol. Nauki* **19**, 40–44 (1976).
10. Binkley, S. A., Riebmman, J. B. & Reilly, K. B. *Science* **202**, 1198–1201 (1978).
11. Semm, P. & Vollrath, L. *J. neural Transmission* **47**, 181–190 (1980).
12. Walcott, C. J. *exp. Biol.* **70**, 105–123 (1977).
13. Semm, P. & Vollrath, L. *Neurosci. Lett.* **12**, 93–96 (1979).
14. Reiter, R. J. *Rev. Physiol.* **35**, 305–328 (1973).
15. Turek, F. W. *Science* **194**, 1441–1443 (1976).
16. Kappers, J. A. Z. *Zellforsch. mikrosk. Anat.* **52**, 163–215 (1960).
17. Demmel, U., Höck, A., Kasperek, K. & Feinendegen, L. E. in *Nuclear Activation Techniques in the Life Sciences*, 193–203 (International Atomic Energy Agency, Vienna, 1979).
18. Krstić, R. *Cell Tissue Res.* **174**, 129–137 (1976).
19. Frankel, R. B., Blakemore, R. P. & Wolfe, R. S. *Science* **203**, 1355–1356 (1979).
20. Leask, M. J. M. *Nature* **267**, 144–145 (1977).
21. Collin, J. P. *Ciba Fdn Symp.* **79**, 120 (1971).
22. Moore, R. Y., Heller, A., Wurtman, R. J. & Axelrod, J. *Science* **155**, 220–223 (1967).

β -Carboline-3-carboxylic acid ethyl ester antagonizes diazepam activity

S. S. Tenen & James D. Hirsch

Department of Biological Research, G. D. Searle & Company,
PO Box 5110, Chicago, Illinois 60680

Analogous to the progression of events in the opiate receptor-enkephalin area, the first reports¹⁻³ that benzodiazepines have selective and specific high-affinity binding sites in brain have stimulated a search for the endogenous 'ligand' or substance that might normally act at these sites⁴⁻⁹. Braestrup and co-workers have extracted from human urine a γ -fraction (ref. 10) which they have recently¹¹ identified as β -carboline-3-carboxylic acid ethyl ester (β CEE). They reported that this substance is extremely potent in displacing ³H-diazepam from brain binding sites and proposed that a β -carboline-3-carboxylic acid derivative might, in part, be the endogenous ligand for the brain benzodiazepine receptor. We have examined several synthetically derived β -carboline-3-carboxylic acid analogues and now present data obtained from testing only the β CEE described by Braestrup *et al.* In addition to confirming these workers' observation that this compound is a potent displacer of ³H-diazepam from brain tissue, our pharmacological data indicate that β CEE has activity that is opposite to, rather than similar to, that of diazepam.

The *in vitro* ³H-diazepam binding assay was carried out in triplicate according to the method of Mackerer *et al.*¹², using whole rat brain membranes. ³H-diazepam was present at 4.0 nM and nonspecific binding was determined in the presence of 4 μ M unlabelled diazepam. Our assay yielded an IC₅₀ for β CEE of 1.9 ± 0.1 nM (mean $\pm$ s.e., eight experiments) which closely corresponds to the reported results¹¹ and is in the range of the most potent benzodiazepines.

Diazepam has anticonvulsant activity and is especially potent in blocking pentylenetetrazole (PTZ)-induced clonic seizures in mice. We determined the PD₅₀ (50% protective dose) of diaze-

Table 2 The effect of either normal saline or β CEE on the CD₅₀ of PTZ

Pretreatments (mg per kg, i.v.)	PTZ dose range (mg per kg, i.v.)	No. of mice	CD ₅₀ of PTZ (mg per kg)
Normal saline	20-35	48	26.0 (23.8-28.5)
β CEE (3.2)	5-20	48	14.3 (12.3-16.8)
β CEE (10)	5-20	48	12.3 (10.3-14.8)
β CEE (32)	1-15	48	8.3 (6.5-10.6)

The subjects were male HAM/ICR CD-1 mice weighing 20-27 g at the time of the experiments. The CD₅₀ (convulsive dose for clonic seizures) of PTZ for each of the four experimental conditions was estimated from four dose levels using 12 mice per dose. The CD₅₀s (and 95% confidence limits) were calculated by a Searle computer program based on the method of Litchfield and Wilcoxon¹³. The lowest and highest doses of PTZ administered to mice in each pretreatment group are also presented. In all four pretreatment groups none of the mice convulsed at the lowest dose of PTZ and 12 out of 12 mice convulsed at the highest dose.

pam against PTZ-induced clonic seizures in mice pretreated with either normal saline or β CEE at 3.2, 10 or 32 mg per kg. The experimental design was as follows: normal saline or β CEE was given intravenously (i.v.); 5 min later DZM was administered intraperitoneally (i.p.), followed after 20 min by i.v. administration of 35 mg per kg PTZ. Pretreatment with β CEE produced a clear and pronounced dose-responsive increase in the PD₅₀ of diazepam against PTZ-induced clonic convulsions (Table 1). This antagonism of the anticonvulsant effect of diazepam is best illustrated by examining the effects of β CEE on a single diazepam dose. For example, diazepam at 1 mg per kg (in the control group) blocked PTZ-induced convulsions in all 10 mice tested. However, when pretreated with only 3.2 mg per kg β CEE there was a complete reversal in that all 10 mice convulsed at this same dose of diazepam.

On the basis of these data, it is tempting to speculate that, analogous to the naloxone-opiate relationship, the β CEE antagonism of diazepam is primarily due to competitive receptor occupancy. However, it seems that a more complex relationship exists. We examined the direct effects of β CEE on the dose (CD₅₀) of PTZ required to induce clonic convulsions. Mice received i.v. administrations of either normal saline or β CEE, 3.2, 10 or 32 mg per kg, and 25 min later various doses of PTZ were given i.v. As compared with controls, β CEE produced a dose-responsive decrease in the amount of PTZ required to induce clonic convulsions (Table 2). However, β CEE is not in itself a convulsant within the dose range we studied; i.v. doses of up to 75 mg per kg have failed to produce any overt signs of convulsions or tremors.

Thus, β CEE has an unusual pharmacological profile. It potently displaces ³H-diazepam from brain membranes, antagonizes the diazepam blockade of PTZ-induced convulsions, lowers the CD₅₀ of PTZ and has no convulsant activity of its own. This compound might constitute a new and unique class of pharmacological activity. In any case, it should be useful in learning more about the functions of the benzodiazepine receptor and its proposed endogenous ligand. It would be premature to infer that benzodiazepines are 'antagonists' rather than 'agonists' of the naturally occurring ligand, despite the pharmacology of β CEE, until the full identification of the endogenous ligand has been made. Even if the endogenous ligand should involve a β -carboline-3-carboxylic acid derivative we would therefore have tested only what might be a portion or analogue of this ligand. Again, borrowing from the opiate literature, we know that slight modifications of the structure of an agonist can result in markedly different pharmacological activity.

We acknowledge the technical assistance of A. Miller for the pharmacological studies, P. Sumner for the binding studies and P. Yonan for synthesizing β CEE.

Table 1 The effect of pretreatment with either normal saline or β CEE on the PD₅₀ of diazepam against PTZ-induced clonic convulsions

Pretreatments (mg per kg, i.v.)	Diazepam dose range (mg per kg, i.p.)	No. of mice	PD ₅₀ of diazepam (mg per kg)
Normal saline	0.1-1.0	50	0.32 (0.23-0.43)
β CEE (3.2)	0.56-5.6	50	2.7 (2.1-3.5)
β CEE (10)	1.0-10	50	3.8 (2.8-5.2)
β CEE (32)	3.2-32	50	12.6 (9.7-16.3)

The subjects were male HAM/ICR CD-1 mice (obtained from Charles River Breeders), weighing 20-27 g at the time of the experiments. The ED₅₀ of diazepam that blocked PTZ-induced clonic convulsions is here referred to as the PD₅₀ of diazepam. The diazepam PD₅₀ for each of the four pretreatment conditions was derived from five doses of diazepam (covering 1 log dose range in quarter-log steps) with 10 mice per dose. The PD₅₀s (and 95% confidence limits) were calculated by a Searle computer program which is based on the method of Litchfield and Wilcoxon¹³. The lowest and highest doses of diazepam administered to the mice in each pretreatment group are also presented. In all four pretreatment groups 10 out of 10 mice convulsed at the lowest diazepam dose and none convulsed at the highest dose. Note that β CEE cannot be dissolved in water; therefore, this compound was administered as a 'suspension' in normal saline with a few drops of PG/Tween-80. All drug concentrations were adjusted so that the mice received 10 ml of this preparation per kg of body weight. Control animals received equal volumes of the saline PG/Tween-80 vehicle. The dorsal tail veins of the mouse were used for all i.v. administrations. Early pilot studies indicated that even the high dose of 32 mg per kg β CEE when administered i.p. failed to show the pharmacological effects reported here.

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1. Squires, R. F. & Braestrup, C. *Nature* **266**, 732-734 (1977).
2. Mohler, H. & Okada, T. *Life Sci.* **20**, 2101-2110 (1977).
3. Braestrup, C., Albrechtsen, R. & Squires, R. F. *Nature* **269**, 702-704 (1977).
4. Colello, G. D., Hockenbery, D. M., Bosmann, H. B., Fuchs, S. & Folkers, K. *Proc. natn. Acad. Sci. U.S.A.* **75**, 6319-6323 (1978).
5. Skolnick, P., Marangos, P. J., Goodwin, F. K., Edwards, M. & Paul, S. *Life Sci.* **23**, 1473-1480 (1978).
6. Ally, A. I. *et al. Neurosci Lett.* **7**, 31-34 (1978).
7. Asano, T. & Spector, S. *Proc. natn. Acad. Sci. U.S.A.* **76**, 977-981 (1979).
8. Mohler, H., Polc, P., Cumin, R., Pieri, L. & Kettler, R. *Nature* **278**, 563-565 (1979).
9. Davis, L. G. & Cohen, R. K. *Biochem. biophys. Res. Commun.* **92**, 141-148 (1980).
10. Nielsen, M., Gredal, O. & Braestrup, C. *Life Sci.* **25**, 679-686 (1979).
11. Braestrup, C., Nielsen, M. & Olsen, C. E. *Proc. natn. Acad. Sci. U.S.A.* **77**, 2288-2292 (1980).
12. Mackerer, C., Kochman, R. L., Bierschen, B. A. & Bremner, S. S. *J. Pharmac. exp. Ther.* **206**, 405-413 (1978).
13. Litchfield, J. T. Jr & Wilcoxon, F. J. *Pharmac. exp. Ther.* **96**, 99-113 (1949).

Most of the coding region of rat ACTH β -LPH precursor gene lacks intervening sequences

Jacques Drouin & Howard M. Goodman

Howard Hughes Medical Institute Laboratory, Department of Biochemistry and Biophysics, University of California, San Francisco, California 94143

The peptide hormones ACTH, β -endorphin, α - and β -melanotropin(MSH) and possibly γ -MSH are synthesized in the pituitary gland by the processing of a 32,000-molecular weight (MW) polypeptide called proopiomelanocortin (POMC)¹⁻⁵. The existence of a further precursor (pre form) to POMC containing an additional N-terminal 'leader' peptide has been suggested by analysis of the *in vitro* translation products of poly(A)-containing RNA from AtT-20 cells, a mouse ACTH-producing cell line of pituitary origin⁶. Nakanishi *et al.*⁷ cloned and sequenced a cDNA copy of the bovine prePOMC mRNA. This sequence confirmed the known structure of the carboxyl half of POMC and revealed the presence of a new MSH-like moiety, γ -MSH, within the 16,000-MW amino half of the precursor (16K fragment). Recent experiments have suggested that this peptide may act in synergy with ACTH to increase corticosterone and aldosterone production *in vivo* and *in vitro*^{8,9}. We have now isolated from a rat genomic DNA library¹⁰ a segment of a DNA encoding most of POMC, using as probe a mouse 144-base pair cloned cDNA fragment encoding β -MSH and β -endorphin¹¹. The cloned rat gene is one of two (or more) closely related POMC genes. The DNA sequence obtained shows that the cloned POMC gene is not interrupted by any intervening sequence (IVS) between the codon for amino acid 19 and the presumptive poly(A) addition site. This region of POMC encodes all the biologically active peptides mentioned above. The DNA sequence encoding the putative γ -MSH and the coding sequence that precedes it are highly conserved between rat and cow. This may indicate an as yet unrecognized biological function(s) for the NH₂-terminal portion of the 16K fragment.

Polyadenylated RNA from the ACTH-secreting mouse cell line AtT-20 was previously used in this laboratory to clone a 144-base pair cDNA fragment encoding most of the β -MSH and β -endorphin sequences¹¹. This mouse cDNA fragment has now been used as a probe to screen¹² a rat genomic DNA library¹⁰ and isolate a fragment of rat DNA encoding POMC. A restriction enzyme cleavage map of the cloned rat DNA fragment was deduced by analysis of the products of double restriction endonuclease digestion and by hybridization of the DNA digests to the cloned mouse cDNA fragment or to single-stranded cDNA synthesized from poly(A)-containing AtT-20 RNA (Fig. 1). A *Xho*I + *Hind*III fragment (1.6 kilobases) contains all the

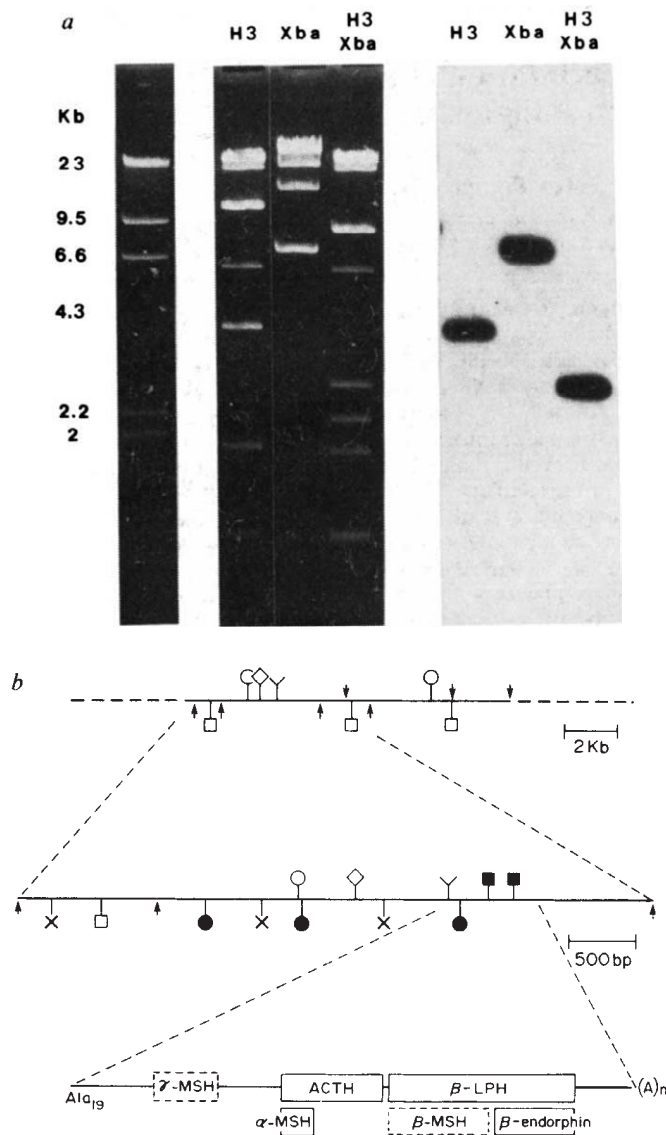


Fig. 1 Restriction enzyme cleavage map of the cloned rat POMC gene. A rat genomic DNA library was constructed by cloning, in the bacteriophage λ vector Charon 4A (ref. 35), fragments of rat (Long-Evans) DNA produced by partial digestion with the restriction enzymes *Hae*III and *Alu*I¹⁰. This library was screened by filter hybridization¹² with the nick-translated¹⁵ 144-base pair mouse cDNA fragment¹¹ and two clones showing positive hybridization were obtained. Restriction mapping analysis showed that the two clones are similar (not shown) and one of them, containing a 12-kilobase insert, was chosen for further work. *a*, The restriction enzyme cleavage map was deduced by analysis of the products of double restriction endonuclease digestion and by hybridization of the digests to the cloned mouse cDNA fragment. This 144-base pair fragment encodes the β -MSH and β -endorphin sequences. DNA (0.5 μ g) was digested with the indicated restriction enzymes and separated by electrophoresis on an 0.8% agarose gel. The gel was stained with ethidium bromide and photographed under short-wave UV light (left panel). The DNA was transferred to nitrocellulose paper¹³ and hybridization was performed as described previously¹⁴ except that 5 $\times$ SSC (1 $\times$ SSC is 0.15 M sodium chloride, 15 mM trisodium citrate) was used in the hybridization solution and 1 $\times$ SSC in the washes (right panel). A probe was prepared by nick translation¹⁵ of the 144-base pair mouse AtT-20 cloned cDNA fragment. DNA size markers³⁶ are provided by a *Hind*III (H3) digest of wild type λ DNA (left lane). The probe hybridizes only to 3.8-kilobase *Hind*III (H3) and 6.7-kilobase *Xba*I (Xba) fragments and to a 2.6-kilobase fragment produced by double digestion with *Hind*III + *Xba*I (H3/Xba). This 2.6 kilobase fragment was later used for sequencing. *b*, Schematic representation of the restriction endonuclease cleavage map obtained for the cloned rat POMC gene. The solid lines represent the restriction map obtained for the cloned rat DNA and the dotted line the Charon 4A vector DNA. The following symbols are used to indicate the sites of restriction endonuclease cleavage: *Hind*III ($\uparrow$), *Eco*RI ($\downarrow$), *Xba*I (∇), *Xho*I (γ), *Hpa*I (*Hinc*II) ($\diamond$), *Bam*HI ($\square$), *Pst*I ($\bullet$), *Acl*I ($\times$) and *Hae*II ($\blacksquare$). All the POMC coding sequences contained in this cloned segment of rat DNA are within the single *Xho*I site (γ) and the *Hind*III site ($\uparrow$) at the far right of the middle line of the figure.

cloned rat sequences that are complementary to the mouse cDNA fragment but a larger (2.6 kilobase) *Xba*I + *Hind*III fragment was later used for sequencing.

The restriction map of the cloned rat POMC gene can be compared with POMC sequences present in rat genomic DNA by the filter hybridization method of Southern¹³. Rat HTC cell DNA was digested with various restriction enzymes, transferred to nitrocellulose filters¹³ and hybridized¹⁴ to the nick-translated¹⁵ mouse cDNA fragment. Most digests show hybridization of unequal intensity to two bands (Fig. 2); however, a consistent restriction map can be independently derived for the strong and weak hybridizing bands. The sizes of the weakly hybridizing fragments correspond exactly to fragments present in the cloned gene. The cloned gene (Fig. 1) will be referred to as POMC I and the other set of fragments as POMC II. The intensity of the bands corresponding to the two POMC genes in the genomic blot (Fig. 2) suggest that POMC II is present in more copies than POMC I. However, in similar experiments with rat liver DNA, bands corresponding to genes I and II were detected and both were of the same intensity (not shown). The difference in intensity found with DNA from HTC cells could well be due to the heteroploidy of these cells¹⁶. We conclude from these results that there are at least two POMC genes in the rat, as is the case for rat insulin^{17,18}.

The rat POMC I DNA sequence was determined (Fig. 3) and it has the same overall organization as the bovine cDNA sequence⁷. The sequence (Fig. 3) is unambiguously oriented with respect to the restriction map (Fig. 1) by the relative position of the *Xho*I and *Pst*I (at amino acids 23–25) sites. The 5' end of the gene is not present in the sequence and is probably not included in the cloned genomic DNA segment. By comparing the rat sequence with the bovine⁷, human¹⁹ and partial rat amino acid sequences⁶, we can deduce that it encodes part of POMC and starts at the 19th codon (alanine) of the precursor (Fig. 3). An IVS interrupts the coding sequence between amino acids 18 and 19 and the junction between this IVS and the coding region is consistent with the consensus sequence proposed for such boundaries²⁰. The agreement between the DNA and the partial amino acid^{6,21} sequences (underlined in Fig. 3), together with the very high homology between the rat and bovine DNA sequences in regions of known biological function, support the idea that the sequenced POMC I gene could be expressed; stronger evidence will be provided when cDNA copies of rat POMC are sequenced. Furthermore, recent work on pulse-labelled rat pars intermedia POMC suggests that two forms of the precursor differing in their amino acid sequence are expressed²².

Comparison of the rat genomic DNA and bovine cDNA sequences reveals that three regions are highly conserved between them: the γ -MSH region (amino acids 19–74), ACTH (amino acids 98–136) and the β -MSH/ β -endorphin region (amino acids 160–209). Not only is the postulated⁷ γ -MSH sequence (position 51–62) highly conserved between rat, beef⁷ and human¹⁹, but so is the sequence preceding it (position 19–48). Furthermore, one pair of basic amino acids that delimits γ -MSH in the bovine sequence⁷ (position 63, 64) is not conserved in the predicted rat sequence, suggesting that if a γ -MSH peptide was made by processing from this precursor it would be 24 amino acids long rather than 12. This is in agreement with the observation that a synthetic 27-amino acid bovine peptide (γ_3 -MSH) shows similar biological activity to tryptic digests of the 16K fragment^{8,9}. These results suggest that the peptide(s) encoded in the γ -MSH region of POMC exert some as yet unrecognized biological function(s).

The predicted rat ACTH sequence is closely homologous to the bovine⁷ and other known ACTH²³ sequence. Worthy of mention is the asparagine at position 126 (position 29 of ACTH) within the sequence Asn-Glu-Ser: this residue is likely to be the site of glycosylation in rat and mouse ACTH^{3,4}.

The predicted rat β -MSH sequence is similar to bovine β -MSH but it does not have the pair of basic amino acids that precedes the bovine sequence. Unless a different type of

processing is involved, this would mean that the only peptides carrying the β -MSH sequence that could be produced from this rat POMC would be the NH₂-terminal fragment of 38 amino acids (γ -LPH) and β -LPH. Human γ - and β -LPH are the only circulating forms of ' β -MSH' immunoreactivity that have been detected²⁴. A similar situation might exist in the rat.

Two regions of POMC have diverged considerably between the rat and bovine sequences: those preceding (that is, within the 16K fragment) and following (the amino end of β -LPH) ACTH. In the first region, the rat sequence encodes 8 amino acids less than the bovine sequence and rat γ -LPH would be 22 amino acids shorter than its bovine⁷ homologue, as suggested by Mains²⁵.

The rat 3'-untranslated region is 60 base pairs shorter than the bovine sequence and only the last 54 base pairs are relatively conserved. This conserved segment contains the sequence AATAAA thought to be involved in poly(A) addition²⁶ and the sequence ACTTCACAGC related to a sequence often observed just before the poly(A) addition site²⁷. A 22-base pair sequence and its almost exact reverse complement (18/22 base pairs) are found at a similar distance from the presumptive poly(A) addition site in the rat and bovine 3'-untranslated regions (indicated by arrows in Fig. 3).

The observation that the bulk of the coding sequence for POMC is uninterrupted by an intervening sequence in the cloned genomic DNA fragment is of interest. The genomic

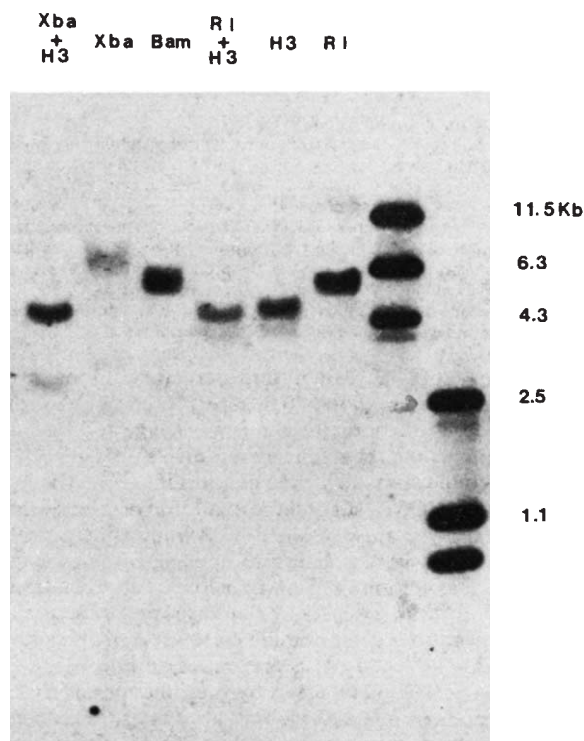
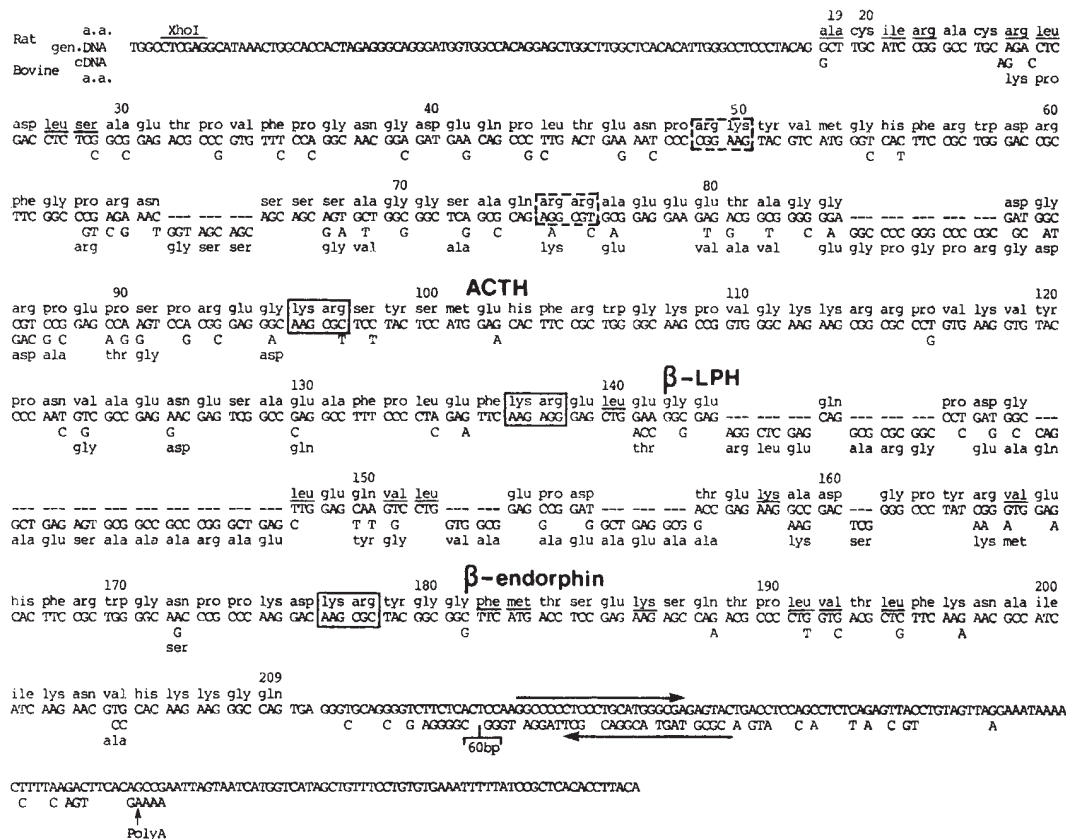


Fig. 2 Genomic DNA hybridization. Rat DNA isolated from HTC cells was digested with restriction enzymes and electrophoresed on an 0.8% agarose gel. The transfer to nitrocellulose and the hybridization were done as described in Fig. 1 legend. The two right lanes contain DNA size markers run on the same gel. They are: *Eco*RI-digested pWB91 (11.5 kilobases)³⁷, *Eco*RI-digested ColE1 (6.3 kilobases), *Eco*RI-digested pBR322 (4.3 kilobases) and the products of pBR322 digestion with *Ava*I and *Hind*III (2,483, 1,104 and 722 base pairs, respectively)³⁸. The 144-base pair mouse cDNA fragment cloned in pBR322 was nick translated¹⁵ and used as probe. *Eco*RI (RI) DNA fragments of 13 and 5.5 kilobases, and two *Hind*III (H3) fragments of 4.4 and 3.8 kilobases, hybridize to the probe. The 3.8-kilobase *Hind*III fragment is not digested by *Eco*RI and corresponds in size and restriction map to the one present in the cloned gene. Both *Bam*HI (Bam) and *Xba*I (Xba) produce broad bands (probably doublets) at about 5.4–5.8 and 6.3–6.7 kilobases, respectively. Another weakly hybridizing *Xba*I fragment of about 2.8 kilobases does not correspond to any fragment found in the cloned gene. Finally, the double digest of *Hind*III + *Xba*I generates a 2.6-kilobase fragment as in the cloned gene and a more intensely hybridizing band at about 4.4 kilobases (Kb).

Fig. 3 DNA sequence of the rat POMC I gene. The DNA nucleotide sequence of the cloned rat POMC gene was determined by the dideoxy chain termination method³⁹ using single-stranded viral DNA from M13mp2 (refs 40, 41) subclones as templates and the synthetic dodecanucleotide d(TCAGCAGTTGT) complementary to the phage sequence as a primer. The 2.6-kilobase *Xba*I/*Hind*III fragment that hybridizes to the cloned mouse cDNA probe (Fig. 1) was digested with one of four restriction enzymes (*Hae*III, *Alu*I, *Hpa*II or *Rsa*I) for subcloning into M13mp2. These restriction fragments were blunt-end ligated into the 'filled-in' *Eco*RI site of the M13mp2 vector, introduced into the appropriate *Escherichia coli* strain by transformation, and clones containing fragments of the POMC gene identified by plaque hybridization¹² with a probe made from the 2.6-kilobase *Xba*I/*Hind*III fragment. Single-stranded DNA was prepared from 1-ml cultures of each positive candidate⁴² and one dideoxy sequencing reaction (ddTTP) was carried out on each to identify similar clones. A representative overlap of different sequence following the *Hae*III of the sequence is therefore predicted translation product of ACTH as in ref. 7 but for polypeptides. The only difference they found a phenylalanine was strain difference; one set of data (solid line) or possible (dashed line).



DNA encoding the functional and structural domains of immunoglobins has been shown to be interrupted by IVSs at the junction between these domains and it was suggested that such gene organization could reflect the evolution of these genes²⁸⁻³⁰. A similar argument was made for the globin genes^{31,32}. The three MSH sequences in POMC suggest the evolution of the precursor by duplications of an ancestral gene. Although there are no evolutionary data it is tantalizing to speculate that the precursor's function as a hormone is responsible for its evolution by duplication rather than by IVS-mediated joining of sequences encoding different structural domains. However, it is possible that the cloned gene lost an IVS present in an ancestral gene. The structural constraints imposed by the function of proteins like the immunoglobulins and globins are very different from those exerted on a hormone precursor like POMC. The addition of a new structural domain to the latter would be very unlikely to result in new hormonal activity because this would require the near concomitant appearance of a new receptor molecule on a target cell. On the other hand, evolution of the hormone precursor by duplication would allow the maintenance of the duplicated segment and its slow drifting to another hormonal activity.

Since submission of this manuscript, A. C. Y. Chang *et al.*³³ have published a genomic DNA sequence encoding the same portion of human POMC. The human gene is interrupted by an IVS at about the same position in the coding region and the human sequence diverges from the bovine in the same regions as the rat DNA sequence. Also, Nakanishi *et al.*³⁴ have published the protein-coding sequence of the bovine ACTH- β -lipotropin precursor gene, and find that it is split near the signal peptide region by an intervening sequence of about 2.2 kilobase pairs.

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1. Roberts, J. L. & Herbert, E. *Proc. natn. Acad. Sci. U.S.A.* **74**, 4826-4830 (1977).
2. Mains, R. E., Eipper, B. A. & Ling, N. *Proc. natn. Acad. Sci. U.S.A.* **74**, 3014-3018 (1977).
3. Eipper, B. A. & Mains, R. E. *J. supramolec. Struct.* **8**, 247-262 (1978).
4. Roberts, J. L., Phillips, M., Rosen, P. A. & Herbert, E. *Biochemistry* **17**, 3609-3618 (1978).
5. Crine, P. *et al. Proc. natn. Acad. Sci. U.S.A.* **75**, 4719-4723 (1978).
6. Gossard, F., Seidah, N. G., Crine, P., Routhier, R. & Chretien, M. *Biochem. biophys. Res. Commun.* **92**, 1042-1051 (1980).
7. Nakanishi, S. *et al. Nature* **278**, 423-427 (1979).
8. Pedersen, R. C. & Brownie, A. C. *Proc. natn. Acad. Sci. U.S.A.* **77**, 2239-2245 (1980).
9. Pedersen, R. C., Brownie, A. C. & Ling, N. *Science* **208**, 1044-1045 (1980).
10. Maniatis, T. *et al. Cell* **15**, 687-701 (1978).
11. Roberts, J. L. *et al. Proc. natn. Acad. Sci. U.S.A.* **76**, 2153-2157 (1979).
12. Grunstein, M. & Hogness, D. S. *Proc. natn. Acad. Sci. U.S.A.* **72**, 3961-3965 (1975).
13. Southern, E. M. *J. molec. Biol.* **98**, 503-517 (1975).
14. Jeffreys, A. J. & Flavell, R. A. *Cell* **12**, 429-439 (1977).
15. Rigby, P. W. J., Dieckmann, M., Rhodes, C. & Berg, P. *J. molec. Biol.* **113**, 237-251 (1977).
16. Thompson, E. B., Tomkins, G. M. & Curran, J. F. *Proc. natn. Acad. Sci. U.S.A.* **56**, 296-303 (1966).
17. Cordell, B. *et al. Cell* **18**, 533-543 (1979).
18. Lomedico, P. *et al. Cell* **18**, 545-558 (1979).
19. Seidah, N. G. *et al. Biochem. biophys. Res. Commun.* **95**, 1417-1424 (1980).
20. Seif, I., Khoury, G. & Dhar, R. *Nucleic Acids Res.* **6**, 3387 (1979).
21. Gianoulakis, C., Seidah, N. G., Routhier, R. & Chretien, M. *Int. J. Peptide Protein Res.* (in the press).
22. Crine P. *et al. Biochemistry* (in the press).

23. Dayhoff, M. O. *Atlas of Protein Sequence and Structure* Vol. 5, D194–D197 (Natn. Biomed. Res. Fdn, Washington, DC, 1972).
24. Bloomfield, G. A., Scott, A. P., Lowry, P. J., Gilkes, J. J. H. & Rees, L. H. *Nature* **252**, 492–495 (1974).
25. Mains, R. E. & Eipper, B. A. in *Endorphins* (eds Graff, L., Palkovits, M. & Ronai, A. Z.) 79–126 (Akademia Kiado, Budapest, 1978).
26. Proudfoot, N. J. & Brownlee, G. G. *Nature* **263**, 211–214 (1976).
27. Benoist, C., O'Hare, K., Breathnach, R. & Chambon, P. *Nucleic Acids Res.* **8**, 127–142 (1980).
28. Gilbert, W. *Nature* **271**, 501 (1978).
29. Darnell, J. *Science* **202**, 1257–1260 (1977).
30. Doolittle, W. *Nature* **272**, 581–582 (1978).
31. Eaton, W. A. *Nature* **284**, 183–185 (1980).
32. Craik, C. S., Buchman, S. R. & Beychok, S. *Proc. natn. Acad. Sci. U.S.A.* **77**, 1384–1388 (1980).
33. Chang, A. C. Y., Cochet, M. & Cohen, S. N. *Proc. natn. Acad. Sci. U.S.A.* **77**, 4890–4894 (1980).
34. Nakanishi, S. *et al.* *Nature* **287**, 752–755 (1980).
35. Blattner, F. R. *et al.* *Science* **196**, 161–169 (1977).
36. Daniels, D. L., de Wet, J. R. & Blattner, F. F. *J. Virol.* **33**, 390–400 (1980).
37. Barnes, W. M. *Science* **195**, 393–394 (1977).
38. Sutcliffe, J. G. *Nucleic Acids Res.* **5**, 2721–2728 (1978).
39. Sanger, F., Nicklen, S. & Coulson, A. R. *Proc. natn. Acad. Sci. U.S.A.* **74**, 5463–5467 (1977).
40. Messing, J., Gronenborn, B., Muller-Hill, B. & Hofschneider, P. H. *Proc. natn. Acad. Sci. U.S.A.* **71**, 3612–3616 (1977).
41. Gronenborn, B. & Messing, J. *Nature* **272**, 375–377 (1978).
42. Winter, G. & Fields, S. *Nucleic Acids Res.* **8**, 1965–1974 (1980).

Selective processing of β -endorphin in regions of porcine pituitary

D. G. Smyth & S. Zakarian

National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA, UK

The prohormone of β -endorphin is unusual in that it is the precursor of more than one biologically active peptide^{1–3} (Fig. 1). The activation of this prohormone, to produce corticotropin (ACTH), α -melanotropin (α -MSH) and β -endorphin, would seem to be relatively complex as its processing pattern is known to differ between tissues. Thus ACTH is produced in the anterior pituitary whereas α -MSH is formed in the pars intermedia^{4,5}; similarly, lipotropin and β -endorphin seem to predominate in the anterior pituitary whereas β -endorphin alone has been thought to be the principal component in the pars intermedia. We report here a study of the distribution of β -endorphin-related peptides in various regions of porcine pituitary. The main products in the anterior pituitary were lipotropin and the potent analgesic form of β -endorphin, whereas the main products in the pars intermedia were the inactive lipotropin C'-fragment and its N-acetyl derivative. Thus the processing of the C-terminal region of the β -endorphin prohormone differs markedly between the two regions of porcine pituitary.

We concentrated on the processing of the C-terminal region of the prohormone by studying the distribution of four peptides related to β -endorphin: the C-fragment of the prohormone

(β -endorphin, lipotropin residues 61–91), the C'-fragment (lipotropin residues 61–87), the α , N-acetyl derivative of the C-fragment and the α , N-acetyl derivative of the C'-fragment^{6–8}. These peptides have a similar molecular size and are generally identified and measured together as a single immunoreactive component^{9,10}. Their biological activities, however, differ strikingly: β -endorphin has potent analgesic properties^{11,12} whereas the C'-fragment is 500 times less potent¹³ and the acetyl peptides are inactive^{8,14}. The processing of the β -endorphin prohormone to produce active or inactive endorphins may therefore have physiological significance and because the processing could vary between tissues it is clearly important that the individual peptides should be differentiated.

We studied the distribution of β -endorphin-related peptides in the anterior pituitary and pars intermedia plus posterior pituitary by extracting the peptides from dissected regions of porcine pituitary glands. The products were resolved by gel filtration, ion exchange chromatography and high pressure liquid chromatography (HPLC). To locate the eluted peptides (and to allow estimation of peptide recovery) the four known β -endorphin-related peptides, obtained by isolation from porcine pituitary^{7,8}, were radioiodinated and trace amounts added to the solvent used to extract the tissues. The peptides resolved by column chromatography were detected and measured by radioimmunoassay (RIA) using an antiserum raised against porcine β -endorphin¹⁵, and further evidence for the identity and concentrations of the peptides was obtained by comparison of elution positions and peak heights on HPLC with those produced by reference peptides. This extensive chromatographic procedure was necessary because of the complexity of the mixture of pituitary peptides, and because each of the four β -endorphin-related peptides reacted with the β -endorphin antiserum¹⁵, which prevented their differential estimation by immunoassay of the tissue extracts without previous fractionation.

Gel filtration of the peptides extracted from the anterior pituitary revealed two fractions which differed in molecular size (Fig. 2a); the first emerged in the position of porcine lipotropin and the second contained peptides that eluted in the position of porcine β -endorphin. Resolution of the β -endorphin fraction by ion exchange chromatography showed that the main immunoreactive component co-chromatographed with ¹²⁵I-labelled porcine β -endorphin (Fig. 3) and its identity was confirmed by comparison with authentic porcine β -endorphin during HPLC. There was, in addition, a small amount (<4% of the β -endorphin) of other immunoreactive peptides which were chromatographically identical to peptides present in high concentration in the pars intermedia. Thus, apart from these minor components, the principal C-terminal fragments of the β -endorphin prohormone in porcine anterior pituitary are lipotropin and β -endorphin, with β -endorphin accounting for nearly 50% of the total immunoreactive material.

Gel filtration of the peptides extracted from the pars intermedia and posterior pituitary showed a negligible amount of

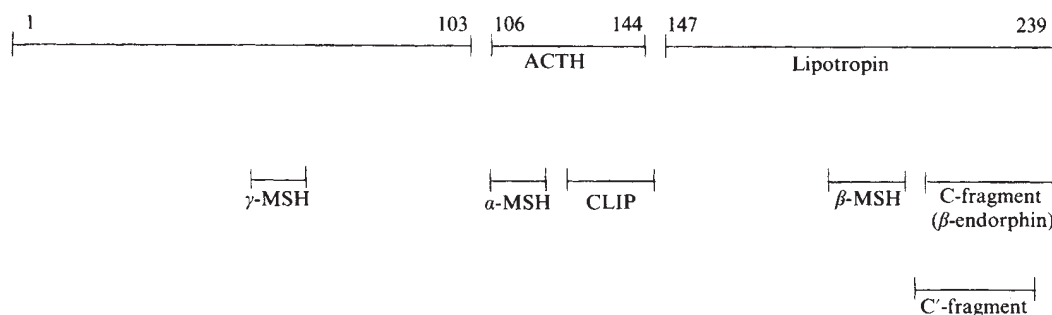


Fig. 1 Schematic representation of the biologically active sections of the 31K prohormone of β -endorphin (according to Nakanishi *et al.*³). The numbers represent residue positions in the sequence of the bovine molecule. Each of the peptides indicated is flanked by pairs of basic residues in the prohormone sequence. Several of the prohormone fragments exist both in acetylated and unacetylated forms⁸.

lipotropin or 31K prohormone, but there was a substantial quantity of immunoreactive peptide with the approximate molecular size of β -endorphin (Fig. 2b). The β -endorphin-containing fraction was resolved into at least six components by chromatography on SP-Sephadex C25 (Fig. 4)—note that the biologically active form of β -endorphin (Peak VI) was only a minor component, representing less than 7% of the immunoreactive peptides. Four of the six peptides (Peaks II, IV, V and VI) co-chromatographed with the corresponding radiolabelled markers and their chromatographic behaviour on HPLC was indistinguishable from the corresponding reference peptides (α , N-acetyl C'-fragment, C'-fragment, α , N-acetyl β -endorphin and β -endorphin). Two of the remaining peptides (Peaks I and III), which were not accompanied by radioactive markers, were found to co-chromatograph on SP-Sephadex C25 and on HPLC with peptides formed by mild reaction of C'-fragment or α , N-acetyl C'-fragment with acetic anhydride *in vitro* and did not correspond to products generated by acetylation of β -endorphin. On this basis the two peptides seem to be an ϵ , N-acetyl derivative of the C'-fragment (Peak III) and an ϵ , N-acetyl derivative of α , N-acetyl C'-fragment (Peak I).

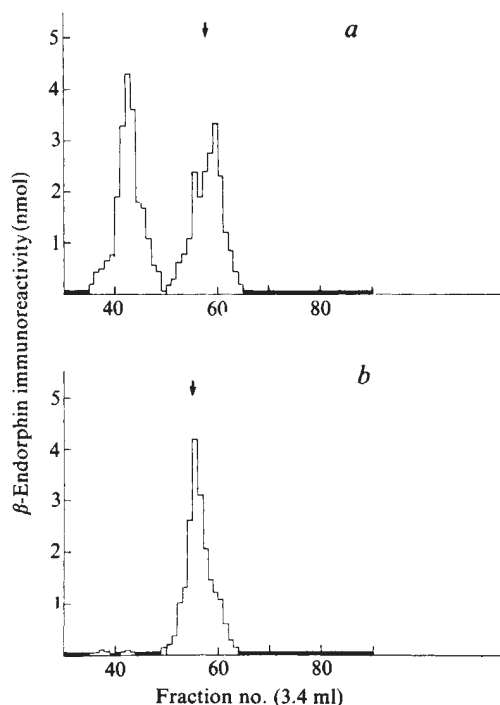


Fig. 2 Gel filtration of peptides related to β -endorphin extracted from *a*, porcine anterior pituitary and *b*, porcine pars intermedia plus posterior pituitary. Acid-acetone extraction (acetone/H₂O/hydrochloric acid, 40/6/1) was carried out at 4 °C on four anterior pituitary lobes or pars intermedia plus posterior pituitary lobes, dissected 20 min *post mortem*. Radiolabelled marker peptides were added before extraction (α , N-acetyl C'-fragment, C'-fragment, α , N-acetyl β -endorphin and β -endorphin) were labelled by treatment of 1 μ g of peptide with 100 μ Ci of ¹²⁵I and $\sim 6 \times 10^5$ c.p.m. of each peptide was used). The extract was centrifuged at 15,000 r.p.m. for 60 min, acetone was removed by concentration *in vacuo* at 20 °C, and the residual solution diluted in 50% acetic acid in preparation for gel filtration on a Sephadex G75 column (70 $\times$ 2.5 cm) in 50% acetic acid. The elution position of the ¹²⁵I-labelled β -endorphin related marker peptides is indicated by an arrow. Aliquots (0.25 μ l) from the anterior pituitary fractions and (0.5 μ l) from the pars intermedia fractions were immunoassayed using an antiserum to β -endorphin: the antiserum did not cross-react with γ -endorphin or methionine enkephalin but reacted with the α , N-acetyl derivative of β -endorphin and the α , N-acetyl derivative of the C'-fragment, with affinity equal to that of the respective unacetylated parent peptides¹⁵.

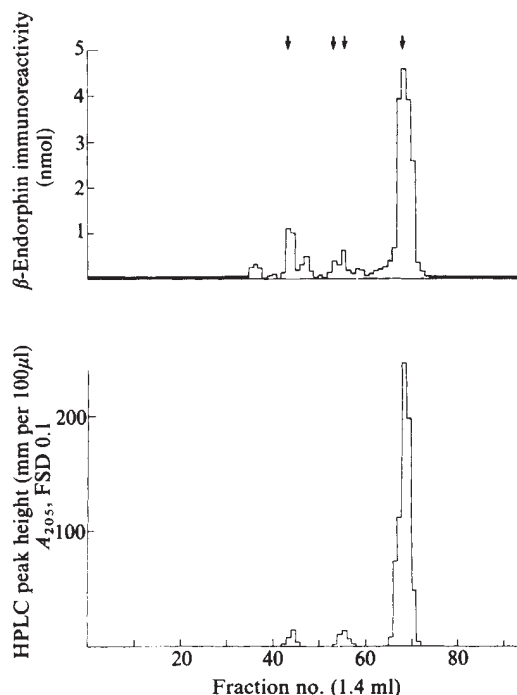


Fig. 3 Ion exchange chromatography of peptides with the molecular size of β -endorphin, obtained by gel filtration of porcine anterior pituitary extracts. After removal of the acetic acid *in vacuo* at 20 °C from the fractions obtained by gel filtration, chromatography of the peptides was performed on a column (60 $\times$ 0.6 cm) of SP-Sephadex C25 in 50% acetic acid with a linear gradient from 0 to 0.6 M sodium chloride, mixer volume 100 ml. The elution positions of the radiolabelled reference peptides (α , N-acetyl C'-fragment, C'-fragment, α , N-acetyl β -endorphin and β -endorphin) are indicated respectively from left to right by the arrows. Aliquots (0.5 μ l) of the column fractions were dried *in vacuo* and immunoassayed with a β -endorphin antiserum¹⁵; the amounts of the peptides determined by RIA shown have not been corrected for their immunoreactivities. A second series of aliquots (20–400 μ l), taken from fractions shown to contain β -endorphin related peptides by RIA, were monitored by HPLC (Waters Associates, Inc.) using a C₁₈ Bondapak column (30 $\times$ 0.4 cm) eluted in 0.01 M hydrochloric acid with a linear gradient (15 min) from 25–40% acetonitrile. The amounts of the peptides shown were determined from the optical density at 205 nm given by the peptide peak occurring at the position of the appropriate unlabelled reference peptide, compared with the peak heights given by known amounts of the marker peptide.

These results show that in the anterior pituitary lipotropin is accompanied by the potent analgesic form of β -endorphin whereas in the pars intermedia the inactive peptides, lipotropin C'-fragment and its N-acetyl derivative, are the major products. Thus the processing of the C-terminal region of the β -endorphin prohormone differs markedly in the two regions.

The relative amounts of β -endorphin and the C'-fragment, together with their acetylated derivatives, may reflect the activity of proteolytic enzymes with specificity for the respective cleavage sites in lipotropin, the Lys-Arg-Tyr residues at positions 59, 60 and 61 and the Lys-Lys-Gly residues at positions 88, 89 and 90. It may be concluded that the specificity of the processing enzymes in the anterior pituitary differs from that in the pars intermedia. Furthermore, the acetylation of β -endorphin and the C'-fragment takes place almost exclusively in the pars intermedia^{16,17}. It is likely that the acetyl endorphins are formed concomitantly with α -MSH because these acetylated peptides occur in the same region of pituitary (ref. 5 and this communication) and originate as fragments of the same prohormone. Note that the acetyl group is important for the melanotropic activity of α -MSH¹⁸ but it renders β -endorphin inactive as an opiate⁸. It seems therefore that activation of one

section of the ACTH-endorphin prohormone can be accompanied by inactivation of another.

In an earlier report¹⁵, we had concluded that the same inactive peptide fragments were present in both the anterior lobe and the pars intermedia of rat pituitary. However, it is now clear that this result was due to slight contamination of the anterior region by cells from the pars intermedia and that the processing patterns are very similar in the corresponding regions of pig and rat pituitary¹⁶. Thus the differential processing mechanisms are conserved across species.

The two regions of porcine pituitary contain approximately equal amounts of the group of β -endorphin-related peptides whereas in the rat the concentration of the peptides in the pars intermedia is nearly two orders of magnitude greater than that in the anterior pituitary. Studies on the significance of these peptides in different species may therefore be useful in obtaining an understanding of the changing physiological role of the pars intermedia during evolution.

The results of this study demonstrate that specific mechanisms lead to the production of β -endorphin in its opiate active form in porcine anterior pituitary. It is known that ACTH is elaborated in the anterior pituitary and is formed from the same prohormone as β -endorphin; thus two biologically active peptides (ACTH and β -endorphin) are generated together with a potential for synergistic action. However, in the pars intermedia, α -MSH is accompanied principally by forms of β -endorphin that have been inactivated by proteolysis or acetylation. It

therefore seems that different processing mechanisms acting on the multifunctional prohormone are available to select the appropriate biological activity required in different regions of the pituitary.

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1. Mains, R. E., Eipper, B. A. & Ling, N. *Proc. natn. Acad. Sci. U.S.A.* **74**, 3014–3018 (1977).
2. Roberts, J. L., Phillips, M., Rosa, P. A. & Herbert, E. *Proc. natn. Acad. Sci. U.S.A.* **75**, 3609–3618 (1978).
3. Nakanishi, S. *et al. Nature* **278**, 423–426 (1979).
4. Lowry, P. J. & Scott, A. F. *Gen. Comp. Endocrinol.* **26**, 16–23 (1975).
5. Jackson, S. & Lowry, P. J. *J. Endocr.* **86**, 205–219 (1980).
6. Bradbury, A. F., Smyth, D. G. & Snell, C. R. in *Peptides: Structure and Biological Activity* (eds Walter, R. & Meienhöfer, J.) 609–615 (Ann Arbor Sci., Michigan, 1975).
7. Smyth, D. G., Snell, C. R. & Massey, D. E. *Biochem. J.* **175**, 261–270 (1978).
8. Smyth, D. G., Massey, D. E., Zakarian, S. & Finnie, M. D. A. *Nature* **279**, 252–254 (1979).
9. Rossier, J. *et al. Proc. natn. Acad. Sci. U.S.A.* **74**, 5162–5165 (1977).
10. Liotta, A. S., Suda, T. & Krieger, D. T. *Proc. natn. Acad. Sci. U.S.A.* **75**, 2950–2954 (1978).
11. Loh, H. H., Tseng, L. F., Wei, E. & Li, C. H. *Proc. natn. Acad. Sci. U.S.A.* **73**, 2895–2898 (1976).
12. Feldberg, W. S. & Smyth, D. G. *Br. J. Pharmac.* **60**, 445–454 (1977).
13. Geisow, M. J., Deakin, J. F. W., Doströvsky, J. O. & Smyth, D. G. *Nature* **269**, 167–168 (1977).
14. Deakin, J. F. W., Doströvsky, J. O. & Smyth, D. G. *Biochem. J.* **189**, 501–506 (1980).
15. Zakarian, S. & Smyth, D. G. *Proc. natn. Acad. Sci. U.S.A.* **76**, 5972–5976 (1979).
16. Smyth, D. G., Zakarian, S., Deakin, J. F. W. & Massey, D. E. in *Peptides of the Pars Intermedia* Ciba Foundation Symp. No. 81 (eds Evered, D. & Lawrenson, G.) (Pitman Medical, in the press).
17. Mains, R. E. & Eipper, B. A. in *Peptides of the Pars Intermedia* Ciba Foundation Symp. No. 81 (eds Evered, D. & Lawrenson, G.) (Pitman Medical, in the press).
18. Schweizer, R. *Ann. NY Acad. Sci.* **297**, 3 (1977).

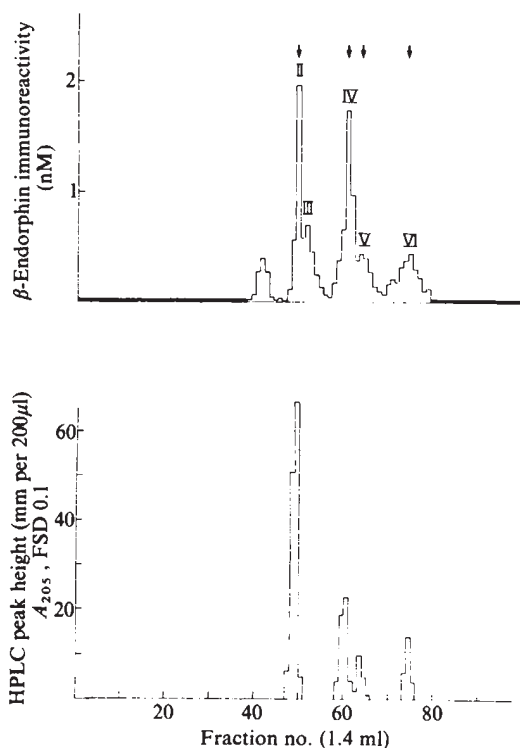


Fig. 4 Ion exchange chromatography of peptides with the molecular size of β -endorphin present in porcine pars intermedia plus posterior pituitary. After gel filtration of the tissue extract on Sephadex G75 in 50% acetic acid, the β -endorphin fraction, localized by RIA and by measuring the radioactivity of the marker peptides, was concentrated *in vacuo* at 20°C and added to a column (60×0.6 cm) of SP-Sephadex C25 in 50% acetic acid. Chromatography was performed as in Fig. 3 legend and the elution positions of the four radiolabelled reference peptides (α , N-acetyl C'-fragment, C'-fragment, α , N-acetyl β -endorphin and β -endorphin) are shown from left to right by arrows. Aliquots (0.5 μ l) of the eluted fractions were dried *in vacuo* and RIA was carried out using the β -endorphin antiserum. A second series of aliquots (20–400 μ l) taken from the fractions that contained β -endorphin immunoreactive peptides, were monitored by HPLC (see Fig. 3 legend) and compared with appropriate reference peptides.

Expression of H-2, laminin and SV40 T and TASA on differentiation of transformed murine teratocarcinoma cells

B. B. Knowles, S. Pan, D. Solter, A. Linnenbach, C. Croce & K. Huebner

The Wistar Institute, 36th Street at Spruce, Philadelphia, Pennsylvania 19104

Murine embryonal carcinoma cells (ECCs) do not express antigens of the major histocompatibility complex (H-2)^{1,2}, but do express cell-surface molecules shared with early embryos³. ECCs are also characterized by their insusceptibility to infection by various oncogenic viruses^{4–7}, and their ability to differentiate into a variety of adult cell types⁸. Differentiation of ECCs *in vitro* can occur spontaneously or can be induced^{9–12}. On exposure to retinoic acid the ECC line F9 (ref. 13) differentiates into cells which have the characteristics of parietal endoderm^{10,14}. When ECCs are exposed to simian virus 40 (SV40), the SV40 tumour (T) antigen is not expressed⁴, although the virus genome reaches the nucleus¹⁵, and a primary transcript of the SV40 A gene is made¹⁶. However, following exposure to retinoic acid, the differentiated cells, like most mouse somatic cells, are susceptible to SV40 abortive infection and synthesize large T and small t antigens¹⁷. To monitor the molecular events associated with the expression of the SV40 A gene on differentiation, we have constructed an ECC line (F9 12-1) containing a single integrated copy of the SV40 genome¹⁸. This was accomplished by introducing a recombinant plasmid consisting of pBR322 linked to the herpes simplex type 1 thymidine kinase gene and SV40 genome¹⁸ into a thymidine kinase-deficient F9 cell line¹⁹. We report here that in F9 12-1 cells exposed to retinoic acid, synthesis of the SV40 A gene product(s), T and tumour-associated specific antigens (TASA), parallels the appearance of the normal hallmarks of differentiation in this cell line, H-2 antigens and the basement membrane protein laminin²⁰.

F9 12-1 cells are morphologically identical to murine ECCs. In our experiments (Table 1), we were unable to find any unequivocally SV40 T antigen-positive nuclei; however, because it is difficult to estimate nuclear fluorescence in ECCs due to their characteristic growth in clumps of rounded cells, we

Table 1 Immunofluorescent assay for detection of antigenic determinants specific to stem cells (SSEA-1), differentiated cells (H-2D^b) and SV40 T antigen

Cell line	Days following exposure to RA	% Positive cells		
		SSEA-1	H-2D ^b	SV40 T antigen*
F9 12-1	0	80	3	0
	2	90	23	2
	4	25	48	11
	7	10	58	50
	9	3	66	65
	11	4	90	70
	15	2	95	80
	16	5	94	90
F9 12-1 clone a (differentiated)		0	100	98
K4RSV		0	100	95
K5RSV		0	0	97

A single-cell suspension of F9 12-1 was placed in 60-mm plastic Petri dishes (Falcon) containing a glass coverslip and cultured in RPMI 1640 with 10% fetal bovine serum (FBS) and 5×10^{-7} M retinoic acid (RA) (*all trans*; Eastman)^{10,14}. The medium was replaced with the same medium without retinoic acid 16 h before assay. F9 12-1 and F9 12-1 clone a were cultured in RPMI with 10% FBS. Cells were resuspended by exposure to 0.25% trypsin in phosphate-buffered saline (PBS) containing 0.1 M EDTA. SSEA-1 expression was determined by indirect immunofluorescent assay using monoclonal antibody to SSEA-1 diluted 1:200 (100 μ l per 2×10^5 cells) as described²¹ elsewhere. Rabbit IgG, anti-mouse IgG (heavy and light chain specific) (RAMIG) tagged with fluorescein isothiocyanate (FITC) (Cappel) served as the developing reagent; cells were mounted in equal parts of Dulbecco's modified PBS and glycerol on glass slides, with coverslips, and viewed using a Leitz Ortholux microscope with epifluorescence and appropriate filters. Cell suspensions were exposed to a 1:100 dilution of monoclonal antibody B22-249R which detects the H-2D^b private specificity 2 (ref. 22). Following exposure to RAMIG-FITC and mounting, cells were scored for fluorescence (see above).

* Coverslips were washed three times with Dulbecco's modified PBS and fixed by 5 min exposure to -20°C acetone and dried. The coverslips were then overlaid with monoclonal antibody to SV40 T antigen, washed and exposed to RAMIG-FITC. Coverslips were mounted in Uvinert aqueous mounting medium (Gurr) and scored for the presence of nuclear fluorescence. In all cases an equivalent amount of IgG from the P3 X63 Ag8 (ref. 23) cell line was used as a control.

cannot exclude the presence of an occasional SV40 T antigen-positive cell. Like all murine ECCs, the F9 12-1 cell does not express H-2D^b (Table 1). Although a very low percentage of the cells are weakly fluorescent following reaction with monoclonal antibody to an H-2D^b antigenic determinant (Table 1), a similar number of fluorescent cells are found when the F9 12-1 cells are incubated with immunoglobulin from the P3X63 Ag8 myeloma cell line. We were also unable to detect those determinants of the H-2K^b or D^b molecules recognized by murine cytotoxic T lymphocytes (CTLs) sensitized by allogeneic immunization (Fig. 1). In addition, no SV40 TASA associated with H-2K^b or H-2D^b and recognized by SV40-sensitized H-2^b-restricted CTLs, are expressed. Murine ECCs are characterized by the expression of a stage-specific embryonic antigenic determinant (SSEA-1) which is also found on murine preimplantation embryos beginning at the 8-cell stage and on cells of the inner cell mass²⁵. SSEA-1 is secreted into the medium of ECCs and can be detected on 80–100% of all murine ECCs tested. F9 12-1 reacts with the monoclonal antibody that defines the SSEA-1 antigenic determinant (Table 1). A small amount of basement membrane protein is synthesized by F9 cell lines²⁶. Antibody to basement membrane protein reacts with a small proportion of cells on the periphery of some of the clumps of the F9 12-1 culture (Fig. 2a). Thus on the basis of morphology, surface-antigen expression and SV40 A gene expression, the F9 12-1 cell seem to behave as other ECCs.

Within 2 days of exposure to retinoic acid some of the F9 12-1 cells assume a flattened, epitheloid morphology. A small percentage of cells express H-2 antigens and the SV40 A gene product(s) T and TASA, are detectable (Table 1, Fig. 1). Most of the cells in these cultures retain the stem cell characteristics. As the cultures are maintained in retinoic acid, differentiation proceeds and after 1 week approximately half the cells morphologically resemble parietal endodermal cells, are synthesizing laminin (Fig. 2b), and express H-2 and SV40 T and TASA.

After 14 days, most of the cells are differentiated, with only a small per cent SSEA-1-positive and over 90% SV40 T- and TASA-positive. These cells also synthesize both laminin and H-2, an indication that cells with the characteristics of parietal

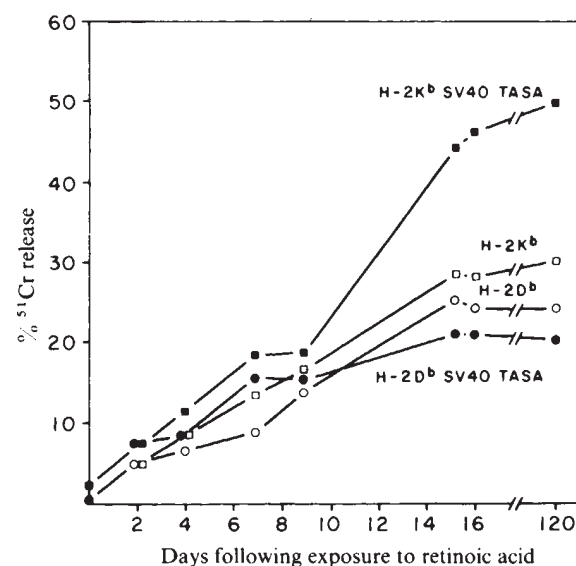


Fig. 1 Detection of H-2K, H-2D and SV40 TASA in association with H-2K^b and H-2D^b in F9 12-1 cells following exposure to retinoic acid. Cells were seeded into 60-mm Petri dishes in RPMI 1640 with 10% FBS and retinoic acid (5×10^{-7} M) (ref. 10). F9 12-1 clone a cells, exposed 120 d previously to retinoic acid, were grown in RPMI 1640 with 10% FBS. Sixteen hours before assay the cells were re-fed with RPMI 1640 and 10% FBS containing ^{51}Cr -labelled sodium chromate (NEZ030; 150 Ci per 5 ml). Control target cells (not shown) were SV40-transformed cell lines derived from C57B 10/ScSn congenic mice; K4RSV (H-2K^bD^b), K5RSV (H-2K^bD^b), LB10SV (H-2K^bD^b) and KD2SV (H-2K^bD^b) (ref. 24). The ^{51}Cr -labelled cells were washed, resuspended and incubated for 7 h at a concentration of 2×10^4 cells per 0.2 ml, with lymphocytes. Percentage ^{51}Cr -release was calculated after γ counting of the supernatant fluid and the cells as described elsewhere²⁴. Cytotoxic effector cells specific for SV40 TASA associated with H-2K^b and H-2D^b were obtained by immunization of B10.A(5R) and B10.A(4R) mice, respectively, with 10^7 infectious units of SV40 in each of the hind footpads. One week after immunization, the popliteal lymph nodes were excised, single-cell suspensions prepared and placed in Dulbecco's modified Eagle's minimal essential medium (supplemented with 10% FBS), 2-mercaptoethanol (2×10^{-5} M), sodium pyruvate (1 mM), Eagle's non-essential amino acids, penicillin and streptomycin) at a concentration of 10^7 cells per 10 ml in 25-cm² plastic tissue culture flasks (Falcon), and incubated at 37°C , 5% CO_2 in humidified air for 3 d before assay²⁴. Effector cells specific for H-2K^b and H-2D^b were obtained by *in vitro* stimulation of BALB/c AnICR (H-2K^dD^d) splenic lymphocytes with irradiated (7,000R) splenic lymphocytes from B10.D2 (R107) (H-2K^bD^b) and B10.HTG (H-2K^dD^b), respectively. BALB/c cells (5×10^6) were incubated with stimulator cells (2×10^6) in 2 ml medium per well of a 24-well Linbro plate. After incubation for 6 d at 37°C in a 5% CO_2 humidified atmosphere, the cells were either re-stimulated *in vitro* (by the addition of 2×10^6 irradiated stimulator cells to 5×10^6 viable cells 2 ml medium) and collected after 5 d or used as effectors immediately. Effector to target cell ratio 10:1 for anti-SV40 TASA effectors and 100:1 for anti-H-2 effectors.

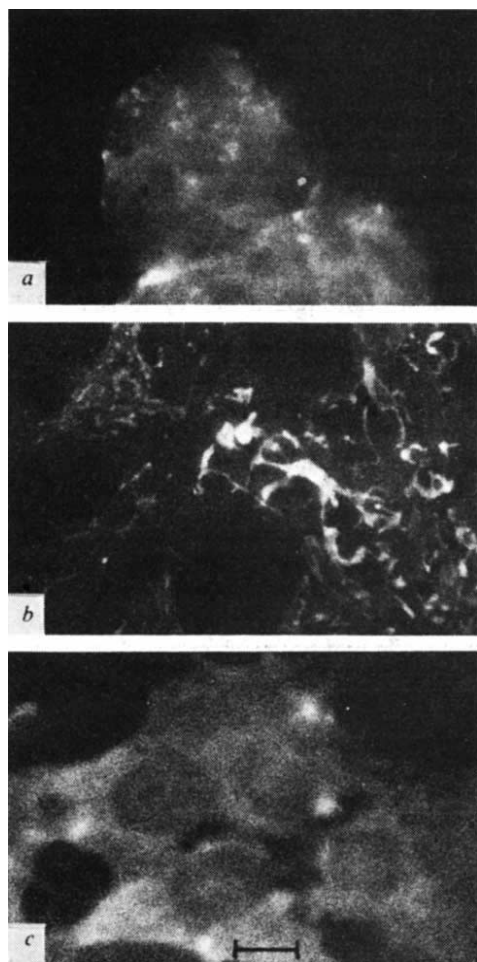


Fig. 2 Immunofluorescent assay for the presence of laminin. Coverslips were fixed (as described in Table 1 legend) and exposed to rabbit anti-mouse basement membrane protein (anti-peak 1) serum²⁷ (provided by Dr Barry Pierce) and absorbed before use with mouse 3T3 cells (1:12 dilution). FITC-tagged goat IgG anti-rabbit IgG served as the developing reagent. *a*, F9 12-1 before exposure to retinoic acid (RA); *b*, 10 d following exposure to RA; *c*, F9 12-1 clone a.

endoderm express antigens of the major histocompatibility complex.

A differentiated cell line, F9 12-1 clone a, which was cloned from retinoic acid-treated F9 12-1 4 months earlier, was included in these experiments. All cells of this clone express H-2, are SSEA-1-negative, T antigen-positive and express SV40 TASA in association with both H-2K^b and H-2D^b. In contrast to the cells monitored for 16 days following retinoic acid treatment, laminin was not detectable in F9 12-1 clone a (Fig. 2c) and the cells appeared fibroblastic. We have previously isolated a differentiated clone (F9 AC clone 9) from retinoic acid-treated F9 cells which has retained its parietal endoderm phenotype for 2 yr in culture. It is possible that, following exposure to retinoic acid, a minor population of fibroblastic cells is always induced and that a representative of these cells gives rise to F9 12-1 clone a. Alternatively, parietal endoderm-like cells may be the only differentiated cells resulting from retinoic acid treatment of F9 cells. However, due to the presence of the SV40 genome, the parietal endoderm phenotype may be transitory with all stable, transformed cells appearing fibroblastic. Experiments are being done to investigate these possibilities.

Recognition of SV40 TASA by virus-specific cytotoxic effector T cells is contingent on expression of the H-2K and D gene products. In contrast to the ECC line PCC3A/1, which does not express H-2 when induced to differentiate by exposure to hexamethylene bisacetamide¹¹, each F9 cell which differen-

tiated cells can independently express the H-2K and H-2D gene observed that somatic cell hybrids between F9 and differentiated cells can independently express the H-2K and H-2D gene products¹⁹, although the mechanism by which this regulatory event occurs remains undefined. Differentiation of the F9 12-1 cell, however, is accompanied by simultaneous expression of both H-2K and H-2D gene products (Fig. 1). This conclusion is based on results with cytotoxic effector cells specific for H-2K^b and H-2D^b. In addition, because recognition of SV40 TASA in mice of the H-2^b haplotype occurs in association with both H-2K^b and H-2D^b (ref. 24), the results with cytotoxic effector cells specific for H-2K^b-SV40 TASA and H-2D^b-SV40 TASA indicate simultaneous expression of the H-2K^b and H-2D^b gene products. Because expression of the H-2 determinants is necessary for CTL recognition of foreign antigens on the cell surface²⁸, it is impossible from inspection of the data in Fig. 1 to determine if the SV40 TASA and H-2 antigens appear simultaneously or sequentially. From the data obtained at 2 and 4 days after retinoic acid induction (Table 1), it can be surmised that the H-2D^b gene product is expressed (and SSEA-1 suppressed) at an earlier time than the SV40 A gene products. However, SV40 T antigen is synthesized in cytoplasm but can only be detected by immunofluorescent techniques following accumulation in the nucleus²⁹. Furthermore, cell-surface antigens are more readily detectable by the methods used, so we feel this difference in time of appearance is artefactual. We therefore propose that, following induction of differentiation, there is simultaneous activation of a series of genes, perhaps through the pleiotropic action of a single gene product (for example RNA splicing enzymes^{16,17}). Alternatively, a single regulatory event could lead to the synthesis of factors necessary for expression of each of the gene products investigated. The organization of the SV40 genome in relation to the cellular genome in the F9 12-1 cell, the F9 12-1 retinoic acid-treated cells and the F9 12-1 clone a cell is identical (A.L. and K.H., unpublished).

Finally Lehman and Friedrich³⁰ report that when ECCs are infected with SV40 and then induced to differentiate, SV40 A gene products are not expressed. Free unintegrated SV40 viral DNA was found in these ECCs, and in the differentiated cells derived from them, that was capable of infecting permissive monkey kidney cells, although integrated viral DNA was not detected. The F9 12-1 cells contain integrated SV40 genomes¹⁸ and when they differentiate the SV40 A gene products are expressed. A comparison of these results suggests that integration of the SV40 genome is a prerequisite for the expression of SV40 gene products.

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- Artzt, K. & Jacob, F. *Transplantation* **17**, 632-634 (1974).
- Forman, J. & Vitteta, E. S. *Proc. natn. Acad. Sci. U.S.A.* **72**, 3661-3665 (1975).
- Solter, D. & Knowles, B. B. *Curr. Topics dev. Biol.* **13**, 139-165 (1979).
- Swartzendruber, D. E. & Lehman, J. M. *J. cell. Physiol.* **85**, 179-188 (1975).
- Boccaro, M. & Kelly, F. *Annls Microbiol. Inst. Pasteur, Paris* **129a**, 227-238 (1978).
- Peries, J., Alves-Cardoso, E., Canivet, M., Debons-Guilemin, M. C. & Lasneret, J. *J. natn. Cancer Inst.* **59**, 463-465 (1977).
- Teich, N. M., Weiss, R. A., Martin, G. R. & Lowy, D. R. *Cell* **12**, 973-982 (1977).
- Martin, G. R. *Cell* **5**, 229-243 (1975).
- Speers, W. C. & Lehman, J. M. *J. cell. Physiol.* **88**, 297-306 (1976).
- Strickland, S. & Mahdavi, V. *Cell* **15**, 393-403 (1978).
- Jakob, H., Dubois, P., Eisen, H. & Jacob, F. *C. r. heb. Séanc. Sci., Paris* **286D**, 109-111 (1978).
- Jetten, A. M., Jetten, M. E. R. & Sherman, M. I. *Expl Cell Res.* **124**, 381-391 (1980).
- Bernstine, E. G., Hooper, M. L., Granchamp, S. & Ephrussi, B. *Proc. natn. Acad. Sci. U.S.A.* **70**, 3899-3903 (1973).
- Solter, D., Shevinsky, L., Knowles, B. B. & Strickland, S. *Dev. Biol.* **70**, 515-521 (1979).
- Swartzendruber, D. E., Friedrich, T. D. & Lehman, J. M. *J. cell. Physiol.* **93**, 25-30 (1977).
- Segal, S., Levine, A. J. & Khoury, G. *Nature* **280**, 335-339 (1979).
- Segal, S. & Khoury, G. *Proc. natn. Acad. Sci. U.S.A.* **76**, 5611-5615 (1979).
- Linnenbach, A., Huebner, K. & Croce, C. M. *Proc. natn. Acad. Sci. U.S.A.* **77**, 4875-4879 (1980).
- Gmur, R., Solter, D. & Knowles, B. B. *J. exp. Med.* **151**, 1349-1359 (1980).
- Howe, C. C. & Solter, D. *Dev. Biol.* **77**, 480-487 (1980).
- Campbell, G. Le M., Goldstein, L. & Knowles, B. B. *J. Cell Biol.* **64**, 719-724 (1975).
- Lemke, H. G., Hammerling, G. J. & Hammerling, U. *Immun. Rev.* **47**, 175-206 (1979).
- Galfre, G., Howe, S. C., Milstein, C., Butcher, G. W. & Howard, J. C. *Nature* **266**, 550-582 (1977).

24. Knowles, B. B., Koncar, M., Pfizenmeier, K., Solter, D. & Trinchieri, G. *J. Immun.* **122**, 1798–1820 (1979).
25. Solter, D. & Knowles, B. B. *Proc. natn. Acad. Sci. U.S.A.* **75**, 5565–5569 (1978).
26. Howe, C. C. & Solter, D. *Dev. Biol.* (in the press).
27. Johnson, L. D. & Starcher, B. C. *Biochim. biophys. Acta* **290**, 158–167 (1972).
28. Doherty, P. C., Solter, D. & Knowles, B. B. *Nature* **266**, 361–362 (1977).
29. Stepleski, Z., Knowles, B. B. & Koprowski, H. *Proc. natn. Acad. Sci. U.S.A.* **59**, 769–776 (1968).
30. Lehman, J. M. & Friedrich, T. D. *J. supramolec. Struct. Suppl.* **4**, 126 (1980).

Post-transcriptional regulation of gene expression in guinea pig tissues

Roger K. Craig*, Ian C. Bathurst†
& David G. Herries‡

*Courtauld Institute of Biochemistry, The Middlesex Hospital Medical School, London W1P 7PN, UK

‡Department of Biochemistry, University of Leeds, 9 Hyde Terrace, Leeds LS2 9LS, UK

The formation of individual functional mRNA sequences in higher organisms requires many steps in addition to transcription. These include RNA splicing, polyadenylation, base modification, transport from nucleus to cytoplasm and assembly into polyribosomes. Various control mechanisms must also operate. These will function on a quantitative basis to account for the differing frequency of the various classes of cytoplasmic mRNAs, and also on a qualitative basis, because in higher organisms not all the nuclear poly(A)-containing RNA molecules are found in a cytoplasmic poly(A)-containing RNA population from the same tissue^{1–10}. During our studies on the mechanisms controlling the accumulation of the poly(A)-containing RNA sequences which occur with high and moderately high frequency in the cytoplasm of the lactating guinea pig mammary gland, it became apparent that >75% of the 20,000 or so poly(A)-containing nuclear RNA sequences were not found in the cytoplasmic poly(A)-containing RNA fraction⁴. Here we demonstrate that many of the poly(A)-containing RNA sequences retained in the nucleus of the lactating guinea pig mammary gland are also present in the nucleus and cytoplasm of the liver of the male guinea pig. These observations provide new evidence for a predominant role of post-transcriptional mechanisms in the regulation of structural gene expression in guinea pig tissue.

Comparison of the base complexity of nuclear and cytoplasmic poly(A)-containing RNA sequences isolated from the lactating guinea pig mammary gland and the male guinea pig liver, demonstrate that the number of different sequences expressed in the nuclear population was similar, but that nearly three times as many poly(A)-containing RNA sequences were present in the cytoplasm of the liver as in the cytoplasm of the mammary gland (Table 1, Fig. 1a). Cross-hybridization studies between the two nuclear poly(A)-containing RNA populations revealed that although each contained similar sequences, there were striking quantitative differences (Fig. 1a). Subsequent hybridization studies using mammary gland nuclear cDNA fractionated into hybridization probes for high- and low-frequency poly(A)-containing RNA sequences showed that sequences present at high frequency in the lactating mammary gland (a group of up to 30 different sequences including the milk proteins^{4,5}) were present at very low frequency in the liver (Fig. 1b). In contrast, the most complex population, consisting of sequences which occurred at low frequency in the lactating mammary gland, were present in the liver in comparable amounts (Fig. 1b).

Cross-hybridization studies using mammary gland⁴ and liver RNA populations⁶ demonstrate that the cytoplasmic poly(A)-containing RNA sequences are all represented within the nuclear poly(A)-containing RNA populations from the same

tissues. This observation, together with the fact that the base complexity of the liver cytoplasmic population is considerably greater than the equivalent lactating mammary gland population suggested that some of those sequences retained in the nucleus of the mammary gland might represent sequences expressed as cytoplasmic poly(A)-containing sequences in liver.

To test this, a cDNA probe representative of those mammary gland poly(A)-containing RNA sequences retained in the nucleus, was hybridized to liver nuclear and cytoplasmic poly(A)-containing RNA. This resulted in maximum hybridization of the probe to nuclear RNA with the expected kinetics, but also significant hybridization to the cytoplasmic RNA population (Fig. 2). We estimate that 3,000–4,000 of those sequences retained in the nucleus of the lactating mammary gland were present in the cytoplasm of the liver. This hybridization cannot be an artefact due to contaminating nuclear sequences within the liver cytoplasmic population because this proportion of the hybridization probe hybridized faster to the liver cytoplasmic poly(A)-containing RNA population than to the liver nuclear poly(A)-containing RNA population. This indicates an enrichment of these sequences within the cytoplasm. Moreover, no significant protection was afforded by a lactating mammary

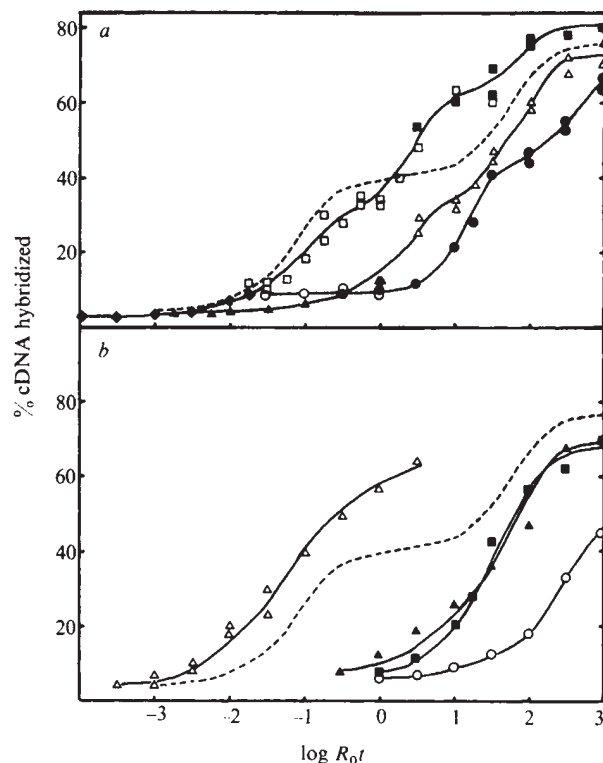


Fig. 1 a, The comparative hybridization kinetics of (1) liver cytoplasmic poly(A)-containing RNA against its homologous cDNA preparation at RNA concentrations of 20 $\mu\text{g ml}^{-1}$ ($\bullet$), 200 $\mu\text{g ml}^{-1}$ ($\square$) and 1 mg ml^{-1} ($\blacksquare$); (2) liver nuclear poly(A)-containing RNA against its homologous cDNA preparation at RNA concentrations of 100 $\mu\text{g ml}^{-1}$ ($\blacktriangle$) and 1 mg ml^{-1} ($\triangle$); and (3) the heterologous hybridization of cDNA prepared from mammary gland nuclear poly(A)-containing RNA against liver nuclear poly(A)-containing RNA at RNA concentrations of 200 $\mu\text{g ml}^{-1}$ ($\circ$) and 1 mg ml^{-1} ($\bullet$). The dashed line represents the hybridization of mammary gland nuclear poly(A)-containing RNA against its homologous cDNA⁴. b, cDNA synthesized from mammary gland nuclear poly(A)-containing RNA was fractionated into high- and low-frequency probes by limited hybridization to a R_0t value of 1.0 mol nucleotide per 1 s followed by separation of double- and single-stranded sequences on hydroxylapatite⁴. The high-frequency cDNA population was then hybridized to either the mammary gland nuclear poly(A)-containing RNA population at an RNA concentration of 100 $\mu\text{g ml}^{-1}$ ($\triangle$), or the equivalent liver population at an RNA concentration of 1 mg ml^{-1} ($\circ$). Low-frequency cDNA probes were hybridized to mammary gland ($\blacktriangle$) or liver nuclear poly(A)-containing RNA ($\blacksquare$), both at an RNA concentration of 1 mg ml^{-1} . The dashed line represents the hybridization of mammary gland nuclear poly(A)-containing RNA against its homologous RNA⁴.

†Present address: Department of Biochemistry, University of Otago, Dunedin, New Zealand.

Table 1 Comparison of the base complexity of nuclear and cytoplasmic poly(A)-containing RNA sequences isolated from lactating guinea pig mammary gland and male guinea pig liver

Source of RNA	Transition	Correct $R_{0t_{1/2}}$	Complexity	No. of species	Relative proportion of total population
Liver	1	$1.16 \times 10^{-2} \pm 4.43 \times 10^{-3}$	$1.86 \times 10^4 \pm 7.07 \times 10^3$	13.3 ± 5.0 [1.55 $\pm$ 0.3]	28 [55]
Post-nuclear fraction	2	$6.47 \times 10^{-1} \pm 1.69 \times 10^{-1}$	$1.03 \times 10^6 \pm 2.70 \times 10^5$	741 ± 194 [31.8 $\pm$ 23.3]	45 [18]
	3	11.64 ± 4.70	$1.85 \times 10^7 \pm 7.50 \times 10^6$	$13,309 \pm 5,395$ [3,242 $\pm$ 1,771]	27 [27]
Liver	1	$3.78 \times 10^{-1} \pm 1.00 \times 10^{-1}$	$6.04 \times 10^5 \pm 1.61 \times 10^5$	387 ± 103 [27.5 $\pm$ 3.7]	38 [49]
Nuclear fraction	2	23.42 ± 3.29	$3.73 \times 10^7 \pm 5.25 \times 10^6$	$23,910 \pm 3,365$ [18,516 $\pm$ 2,425]	62 [51]

Poly(A)-containing RNA was isolated from total nuclear or cytoplasmic RNA, and ^3H -labelled cDNA synthesized from each population (specific activity 1.45×10^7 c.p.m. per μg) using avian myeloblastosis virus reverse transcriptase. Hybridizations were performed in RNA excess and the degree of hybridization determined using S_1 nuclease digestion. Estimations of RNA complexity were based on the comparative hybridization kinetics of a rabbit globin mRNA standard after best line fit by computer and the appropriate adjustment of the observed $R_{0t_{1/2}}$ values (mol nucleotide per l s). Estimation of the number of sequences in each transition for the liver RNA populations was based on a number average molecular size of 1,590 and 1,390 nucleotides for the nuclear and post-nuclear populations, respectively, as determined by sucrose gradient centrifugation in the presence of formamide. Values in square brackets represent numbers previously calculated for the lactating mammary gland⁴. All procedures were as described previously^{4,5,7}. Subsequent best line fits using fractionated cDNA probes in homologous or heterologous hybridizations have been assessed by eye (see Figs 1, 2).

gland cytoplasmic poly(A)-containing RNA population, thereby demonstrating the specificity of the cDNA probe.

The results reveal a remarkable degree of sequence homology within the nuclear poly(A)-containing RNA sequences isolated from two different tissues, one from a male and one from a female animal of the same species. Quantitative differences were also evident, but were restricted to the high-frequency poly(A)-containing RNA sequences. Thus, in common with studies on the distribution of globin¹¹⁻¹⁵ and ovalbumin¹⁶ mRNA sequences, the RNA sequences found at high frequency in mammary gland tissue^{4,5,7}, were present at very low frequency in a nuclear poly(A)-containing RNA population and could not be detected in a cytoplasmic poly(A)-containing RNA population isolated from a different tissue within the same species⁶. The accumulation of these sequences seems to be regulated in a different manner from the complex low-frequency poly(A)-containing RNA sequences.

Our observations have a parallel. In sea urchins, post-transcriptional mechanisms seem to predominate, because transcribed single-copy nuclear sequences are common to embryo and adult tissues⁸, but cytoplasmic mRNA populations differ considerably^{9,17}. We have investigated two tissues, one of which, the liver, contains a relatively heterogeneous cell population¹⁸ compared with the mammary gland, which is predominantly epithelial¹⁹. This disparity in cell type presumably accounts for

the increased complexity of the liver cytoplasmic poly(A)-containing RNA population, thereby reflecting different functional requirements of different cell types even within the low-frequency populations. About 75% of the mammary gland low-frequency cytoplasmic poly(A)-containing RNA sequences are also present in the equivalent liver subcellular fraction (I.C.B. and R.K.C., unpublished). Some of these will direct the synthesis of the 'house-keeping' proteins, those proteins required by all cells^{9,20,21}, others will represent cell-specific RNA sequences from cell types common to both tissues. The remainder, those not found in the liver, must represent a class of low-frequency poly(A)-containing RNA sequences peculiar to the mammary gland.

Overall, our studies support the concept that post-transcriptional mechanisms determine the accumulation of the low-frequency class of poly(A)-containing RNA sequences within the cytoplasm of guinea pig tissues. Thus although this population of sequences when located in the nucleus of two different tissues is very similar, examination of the relative distribution of these sequences within the cytoplasm reveals considerable tissue-specific differences. The precise nature of these post-transcriptional mechanisms has not been established, but probably involves correct processing of the primary RNA transcripts²²⁻²⁴, resulting ultimately in tissue- or cell-specific expression in the cytoplasm.

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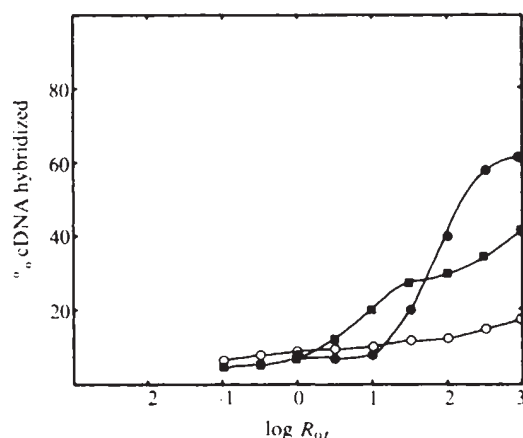


Fig. 2 A mammary gland cDNA probe representative of those sequences confined to the nucleus was prepared by hybridizing the cDNA preparation synthesized from nuclear poly(A)-containing RNA with a post-nuclear poly(A)-containing RNA population to a R_{0t} value of 100 mol nucleotide per l s. Single-stranded cDNA (65% of starting material) was recovered by hydroxylapatite column chromatography⁴, then used as a source of mammary gland nucleus-specific cDNA. This was hybridized to poly(A)-containing RNA from the mammary gland cytoplasmic fraction (○), the liver nuclear (●) and liver cytoplasmic fraction (○), all at an RNA concentration of 1 mg ml⁻¹.

- Davidson, E. H. & Britten, R. J. *Science* **204**, 1052-1059 (1979).
- Lowenhaupt, K. & Lingrel, J. B. *Cell* **14**, 337-344 (1978).
- Guyette, W. A., Matusik, R. J. & Rosen, J. M. *Cell* **17**, 1013-1023 (1979).
- Bathurst, I. C., Craig, R. K., Herries, D. G. & Campbell, P. N. *Eur. J. Biochem.* **109**, 183-191 (1980).
- Bathurst, I. C., Craig, R. K., Herries, D. G. & Campbell, P. N. *Biochem. J.* **192**, 489-498 (1980).
- Bathurst, I. C. thesis, Univ. London (1980).
- Craig, R. K., Boulton, A. P., Harrison, O. S., Parker, D. & Campbell, P. N. *Biochem. J.* **181**, 737-759 (1979).
- Kleene, K. C. & Humphreys, T. *Cell* **12**, 143-155 (1977).
- Galau, G. A. *et al.* *Cell* **7**, 487-505 (1976).
- Balmain, A., Minty, A. J. & Birnie, G. D. *Nucleic Acids Res.* **8**, 1643-1660 (1980).
- Gilmour, R. S., Harrison, P. A., Windass, J. D., Affara, N. A. & Paul, J. *Cell Differentiation* **3**, 9-22 (1974).
- Groudine, M. & Weintraub, H. *Proc. natn. Acad. Sci. U.S.A.* **72**, 4464-4468 (1975).
- Humphries, S., Windass, J. & Williamson, R. *Cell* **7**, 267-277 (1976).
- Gottesfeld, J. M. & Partington, G. H. *Cell* **12**, 953-962 (1977).
- Perlman, S. M., Ford, P. J. & Rosbash, M. M. *Proc. natn. Acad. Sci. U.S.A.* **74**, 3835-3839 (1977).
- Tsai, S. Y., Tsai, M. J., Lin, C.-T. & O'Malley, B. W. *Biochemistry* **18**, 5726-5731 (1979).
- Lev, Z. *et al.* *Dev. Biol.* **76**, 322-340 (1980).
- Van Berkel, T. J. C. *Trends biochem. Sci.* **4**, 202-205 (1979).
- Pitelka, D. R. in *Lactation* Vol. 4 (ed. Larson, B. L.) 41-66 (1978).
- Axel, R., Feigelson, P. & Schutz, G. *Cell* **7**, 247-254 (1976).
- Hastie, N. D. & Bishop, J. O. *Cell* **9**, 761-774 (1976).
- Wickens, M. P., Woo, S., O'Malley, B. W. & Gurdon, J. B. *Nature* **285**, 628-634 (1980).
- Hamer, D. H. & Leder, P. *Cell* **18**, 1299-1302 (1979).
- Early, R. *et al.* *Cell* **20**, 313-319 (1980).

The higher order structure of chicken erythrocyte chromosomes *in vivo*

J. P. Langmore* & C. Schutt

MRC Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH, UK

Recently eukaryotic chromosomes have been shown to consist of a repeating subunit, called the nucleosome¹. Although electron microscopy, neutron scattering and X-ray diffraction have been used to determine the low resolution structure of the nucleosome, these techniques have yielded little information about the disposition of nucleosomes within chromosomes. Electron microscopy has produced many models for chromosome structure based on uniform fibres of 150–500 Å diameter or on globular 'superbeads'^{2–6}. Unfortunately the models are based on microscope images that fail to reveal the strong structural periodicities shown by X-ray scattering to be characteristic of isolated chromatin in solution. Moreover it has not been demonstrated that the chromosomes of living cells are composed of such fibres. We have used low-angle X-ray scattering to investigate the organization of chromosomes *in vivo* and to account for the previously observed inconsistencies in many X-ray and electron microscope observations. We report here that chicken erythrocytes have a 400 Å periodicity due to a nuclear structure that is directly related to the 300 Å side-by-side packing of chromosome fibres revealed by electron microscopy of embedded cells, and that this periodicity can be preserved in isolated nuclei provided that the proper buffers are used.

Studies of concentrated isolated chromatin by X-ray and neutron scattering have shown dominant structural periodicities at about 110, 55, 38, 27 and 22 Å^{7–14}. The intensities and exact positions of these bands depend on ionic composition of the solution and chromatin concentration. Significantly these low-angle studies have not revealed the 150–500 Å packing bands predicted by all the existing models for chromatin. However, the 110 Å band has been used as indirect evidence that helical 300 Å thick fibres exist^{13,14}. Studies of dilute isolated chromatin have shown variable 400–460 Å, weak 200 Å, and weak variable 140 Å periodicities in one case¹⁵, and absent 400 Å, absent 200 Å, strong 110 Å, absent 55 Å, and strong 33 Å in another case¹⁴. Unfortunately, solution studies cannot be definitive because partial nuclease digestion and sub-physiological ionic strength are required for chromatin solubility. In fact, as the ionic strength is raised, increasingly compact higher order structures are formed^{16–18}.

A typical diffraction pattern from living chicken erythrocytes is shown in Fig. 1a. A shoulder in the intensity is apparent at a spacing of ~400 Å. Patterns from rabbit enucleated erythrocytes do not give rise to this shoulder and possess only low-angle scattering from the concentrated intracellular haemoglobin (Fig. 1b). Buffer alone produced only uniform scattering of low intensity (Fig. 1c). By subtracting the measured intensities of nucleated from enucleated erythrocytes, we produced a difference curve (Fig. 2a) representing the structural differences (presumably nuclear) between these cells. A strong 400 Å peak is present in this curve.

The nuclear origin of the 400 Å peak is demonstrated by diffraction from chicken erythrocyte nuclei isolated by lysis of the cells with MLB buffer (see legend to Fig. 1) of near-

physiological ionic strength and containing magnesium (Figs 1f, 2b). The isolated nuclei also scatter at 200, 106, 60, 38 and 30 Å (Figs 1d, e, 2b, 3a). The nuclear spacings smaller than 400 Å were not obvious in the patterns from whole erythrocytes, due to the reduced contrast and increased background from the high internal concentration of haemoglobin. Diffraction patterns from live chicken and mouse lymphocytes, MOPC-21 and HeLa tissue culture cells, sea urchin, scallop and frog sperm cells, and from nuclei of all these cells clearly show the 106, 60, 38, 30 and 22 Å bands to be common to whole cells as well as their nuclei. Further discussion of these results will be published elsewhere.

We are confident that the 400 Å band is of chromosomal origin because it is eliminated by nuclease treatment (Fig. 3a). Also thin sections of embedded erythrocytes reveal periodicities of comparable spacings only in the chromocentres (condensed regions of chromatin) and a lack of extraneous cytoplasmic and nuclear structures such as ribosomes, microtubules, mitochondria and nucleoli, which might in other cell types give rise to non-chromosomal low-angle bands. However, electron microscopy of chicken erythrocytes⁴ and of isolated nuclei (unpublished results) indicate that the chromocentres have a granular internal structure with a periodicity of only 300 Å, apparently composed of square-packed thick fibres of ~300 Å diameter.

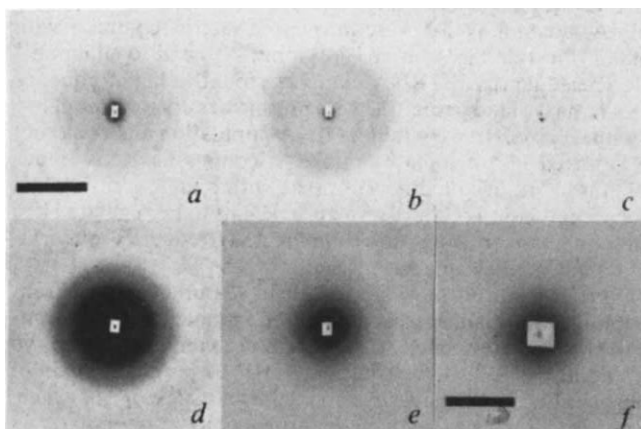


Fig. 1 Low-angle X-ray diffraction patterns recorded on a Franks²² camera with two 20-cm bent-glass mirrors and a 33.3-m specimen-to-film distance, mounted on a GX-13 rotating anode X-ray generator (Marconi-Elliott Avionics) producing 1.54 CuK α nickel-filtered radiation. The specimens were cooled to 5°C and continuously translated over 2–3 cm during the X-ray exposure. The patterns were recorded on Ilford G film and uniformly developed. Aliquots of blood (1 ml, freshly collected with 10 unit ml⁻¹ heparin) were washed three times by centrifugation at 100g for 5 min in 10 ml of wash buffer (consisting of 130 mM NaCl, 5 mM KCl, 2 mM MgCl₂ and 10 mM HEPES, pH 7.3) and centrifuged into capillaries at 100g. X-ray exposure caused no noticeable haemolysis or uptake of trypan blue. Nuclei were prepared by lysis and resuspension of the pelleted cells three times for 3 min in 10 ml of MLB (consisting of 60 mM KCl, 15 mM NaCl, 15 mM HEPES, pH 7.3, 2 mM MgCl₂, 0.1% Nonidet P-40 and 1 μ M fresh phenylmethylsulphonyl fluoride (PMSF)) and centrifuged into capillaries at 1,000g. SDS-polyacrylamide gel electrophoresis of nuclei after X-ray exposure showed no evidence of histone proteolysis. *a*, 4.2 h exposure from living chicken erythrocytes, *b*, 4.5 h exposure from living rabbit erythrocytes. *c*, 16.5 h exposure from wash buffer in capillary. *d*, Pattern from isolated chicken erythrocyte nuclei reproduced to illustrate the 60 Å and 38 Å bands. *e*, Pattern from isolated chicken erythrocytes reproduced to illustrate the 106 Å band. *f*, Pattern from isolated chicken erythrocytes (recorded at 44 cm specimen-to-film distance using Kodak Industrex AA film) reproduced to illustrate the 400 Å band. Length of the bar in *f* represents 1/50 Å⁻¹ in *a–e*. Bar in *f* represents 1/200 Å⁻¹ in *f*. *a–c* have been identically reproduced. The 400 Å shoulder is almost as visible by eye as the 106 and 60 Å features. The 200 Å shoulder is very weak and very difficult to visualize in photographic prints. The entire series of reflections from nuclei was consistently observed in more than 20 preparations using various conditions, including pH in the range 6–8, in the presence of 15 mM 2-mercaptoethanol, and in the presence of 1 mM CaCl₂. Very small amounts of proteolysis eliminate the 400 Å shoulder.

* Present address: Division of Biological Sciences and Biophysics Research Division, The University of Michigan, Ann Arbor, Michigan 48109.

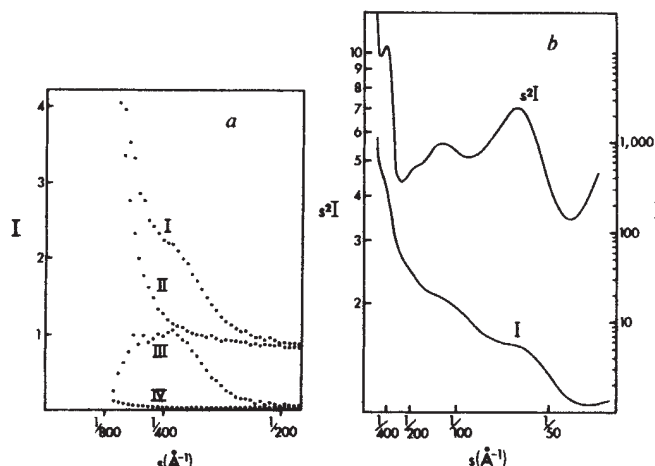


Fig. 2 Quantitative analysis of erythrocyte diffraction patterns. *a*, Measured chicken erythrocyte intensities (from the pattern shown in Fig. 1*a*); curve I, measured rabbit erythrocyte intensities (from the pattern shown in Fig. 1*b*); curve II, the difference between chicken and rabbit erythrocyte intensities; curve III, measured camera and solvent background (from the pattern shown in Fig. 1*c*) normalized to 4.3 h exposure time; curve IV, the mode of the difference curve is at ~ 380 Å, and the mean is at ~ 410 Å. *b*, The measured intensities from chicken erythrocyte nuclei in MLB showing a diffraction pattern on a steep background. I, average scattered intensity and s , $2 \sin(\theta/2)/\lambda$ where θ is the scattering angle and λ the wavelength. By multiplying the recorded intensities by s^2 we can correct for the random orientation of the fibres with respect to the X-ray beam, and for the high specimen background that seems to decrease as $1/s^2$, thereby calculating the true relative strengths (power) of the structural periodicities in the specimen. The chicken intensities are normalized to the rabbit intensities at 0.005 – 0.006 Å $^{-1}$ using a multiplicative factor of 1.3 to correct for the slightly different cell concentrations in the two samples. In this angular range less than 10% of the scattering originates from the nuclei, as estimated by measurements of the steeply falling scattering from isolated nuclei. Two-dimensional densitometry was carried out using a 'Photoscanner' (Optronics) controlled by an interactive computer program. A 50 - μ m step size (equivalent to steps of $s = 0.000098$ Å $^{-1}$) was used. Specular optical densities in the range 0 – 2 were measured and related to X-ray intensities using data applicable to Ilford G film²³. The intensities at the centre of each pattern were displayed to allow the operator to determine the centre of scattering and to exclude unwanted areas of film from analysis. The scattering centre was established by briefly exposing the film to the direct X-ray beam, by moving the beam stop to one side after recording the diffraction pattern. The accuracy of this centre was checked by comparing the distribution of intensity with radius from above and below, and from left and right, of the centre. The centre was refined until the centre of analysis was to within one half raster unit (25 μ m) of the scattering centre. To exclude unnecessary camera background and the beam stop shadow on each pattern, a rectangular area covering these unwanted areas was excluded from analysis. Non-excluded data points in the two horizontal quadrants were averaged within annuli of different radii to give the average intensity distribution as a function of radius. The existence of the 400 Å band was also confirmed by the visual appearance of a 400 Å shoulder on all three films in the film pack and by the presence of the 400 Å shoulder in one-dimensional densitometer measurements.

To establish the relevance of the X-ray spacing *in vivo* and *in vitro* to the microscopical structures, we recorded diffraction patterns during the different preparative steps for microscopy. Although fixation and uranyl acetate staining caused a slight expansion of the structure, ethanol dehydration and embedding in epoxy caused broadening and progressive shrinking of the spacing to 310 Å (Fig. 3*b*). This indicates that the apparent side-by-side partially ordered packing of 300 Å fibres observed by Davies⁴ and us in thin sections is probably directly correlated to the more orderly packing of 400 Å fibres *in vivo*. In addition, dehydration distorts the internal structure of the fibres as shown by loss of the 106 and 60 Å bands and appearance of a broad 70 Å band (Fig. 3*a*), consistent with earlier studies of dehydration^{10,12}.

When chromocentres are dispersed by replacing the $MgCl_2$ (2 mM) of MLB with 2 mM EDTA a homogeneous distribution of thick fibres within the nuclei is seen by electron microscopy and the 400 Å band disappears—all the other reflections are preserved (manuscript in preparation). This substantiates our hypothesis that the 400 Å spacing is from the packing of

nucleofibres and that the 200 , 106 , 60 , 38 , 30 and 22 Å periodicities reflect the internal structure of the fibres.

Do our results resolve any long-standing problems in the interpretation of chromatin X-ray patterns? Our consistent observation of a 106 Å band in each cell type and nucleus examined establishes this periodicity as characteristic of intact chromosomes. Previous investigators have been uncertain that this was an invariant feature of chromatin, especially in nuclei^{7,8,10,19,20}. We have consistently found the 400 Å band present in chicken erythrocytes and erythrocyte nuclei and absent when the fibres are dispersed, which shows that this band is due only to fibre packing and not to periodicities within the fibres themselves. This contradicts Bram and collaborators who conclude that there is a concentration-independent 300 – 450 Å periodicity in isolated chromatin fibres^{15,20}. Furthermore these investigators were unable to find the characteristic 106 Å band in isolated chromatin or nuclei, perhaps due to their use of very low ionic strength buffers.

The reflections seen in whole cells and isolated nuclei have been difficult to visualize due to their diffuse nature and steep background scattering. The lack of sharpness of the bands was not a preparation artefact, but was due to apparent disorder in chromosome structure. The width of the 400 Å band is probably due to heterogeneity or disorder in the packing of the nucleofibres or because the fibres are organized in small domains. Certainly chromosome fibres do not pack with the high degree of long-range crystalline order found in skeletal muscle or collagen. However, even after embedding in plastic, which causes considerable broadening of the packing band, nucleofibres show a degree of packing order easily recognized in electron micrographs. The diffuse nature of the 200 , 106 , 60 , 38 , 30 and 22 Å peaks is not inconsistent with a highly regular nucleofibre internal structure, because in the absence of excellent packing order there are several factors that result in broad bands, including inherent diffuseness of the molecular Fourier transform as in the case of the Bessel functions that describe diffraction from helical fibres, and also random fibre orientation, which leads to overlap of bands at similar angles. In fact, diffraction from the nucleofibre internal structure is no more diffuse than diffraction from randomly oriented double-stranded DNA in solution or in chromatin^{8,21}. The presence of a

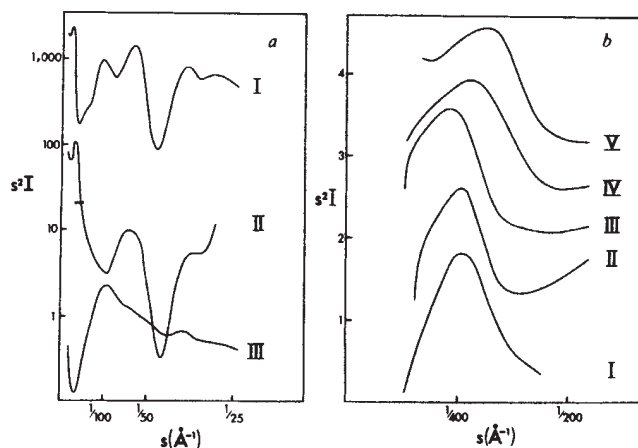


Fig. 3 Effects of dehydration and nuclease digestion on X-ray scattering. *a*, Chicken erythrocyte nuclei in MLB (curve I), after embedding in Araldite (curve II), and after nuclease digestion (curve III). For embedding, nuclei were fixed in 1% glutaraldehyde for 10 min at 0° C, washed in H_2O , stained in 1% UO_2 for 10 min, dehydrated with increasing concentrations of ethanol, propylene oxide and then Araldite, followed by polymerization at 60° C for 2 d. Nuclei at a concentration of about 100 A $_{260}$ per ml were digested at 37° C for 15 min with 5,000 units of micrococcal nuclease and 300 units of DNase I. *b*, Diffraction patterns from chicken erythrocyte nuclei. Curve I, *in vivo* from Fig. 2*a*; curve II, isolated nuclei in MLB; curve III, isolated nuclei in water after fixation and staining as above; curve IV, fixed and stained nuclei in ethanol; curve V, fixed and stained nuclei embedded in Araldite. All curves are arbitrarily positioned on the ordinate in order to minimize overlap, and are therefore not meant to portray the relative intensities but to show the positions of the maximum and minimum in the $s^2 I$ function.

large amount of background scattering in the patterns from living cells and nuclei is not surprising because the fibres are not homogeneously distributed in the sample, but are concentrated in small chromocentres that strongly scatter X rays much as they do visible light. Centrifugation of the nuclei into a transparent pellet eliminates most of this background.

Although the local packing of nucleosomes within chromosomes is conserved among chicken erythrocytes, mouse lymphocytes, HeLa and MOPC-21 cells and several chromatin-containing sperm (as revealed by constancy of the 106, 60, 38, 30 and 22 Å spacings) higher order structure seems to be cell

dependent. For example, lymphocytes have a 330 Å rather than 400 Å periodicity, whereas sperm seem to lack a higher order spacing in the 300–500 Å range (unpublished data). These results suggest that variations in chromosome folding may involve only subtle changes in the local packing of nucleosomes.

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1. Felsenfeld, G. *Nature* **271**, 115–122 (1977).
2. Ris, H. & Kubai, D. F. A. *Rev. Genet.* **4**, 263–294 (1970).
3. Davies, H. G. J. *Cell Sci.* **3**, 129–150 (1968).
4. Walmsley, M. E. & Davies, H. G. J. *Cell Sci.* **17**, 113–139 (1975).
5. Finch, J. T. & Klug, A. *Proc. natn. Acad. Sci. U.S.A.* **73**, 1897–1901 (1976).
6. Renz, M., Nehls, P. & Hozier, J. *Proc. natn. Acad. Sci. U.S.A.* **74**, 1879–1882 (1977).
7. Luzzati, V. & Nicolaieff, A. *J. molec. Biol.* **1**, 127–133 (1959).
8. Wilkins, M. H. F., Zubay, G. & Wilson, H. R. *J. molec. Biol.* **1**, 179–185 (1959).
9. Luzzati, V. & Nicolaieff, A. *J. molec. Biol.* **7**, 142–163 (1963).
10. Nicolaieff, A. in *Small Angle X-Ray Scattering* (ed. Brumberger, H.) 221 (Gordon and Breach, New York, 1967).
11. Garrett, R. A. *Biochim. biophys. Acta* **246**, 553–560 (1971).

12. Pooley, A. S., Pardon, J. F. & Richards, B. M. *J. molec. Biol.* **85**, 533–549 (1974).
13. Sperling, L. & Klug, A. *J. molec. Biol.* **112**, 253–263 (1977).
14. Carpenter, B. G., Baldwin, J. P., Bradbury, E. M. & Ibel, K. *Nucleic Acids Res.* **3**, 1739–1746 (1976).
15. Bram, S., Butler-Browne, G., Baudy, P. & Ibel, K. *Proc. natn. Acad. Sci. U.S.A.* **72**, 1043–1045 (1975).
16. Suau, P., Bradbury, E. M. & Baldwin, J. P. *Eur. J. Biochem.* **97**, 593–602 (1979).
17. Campbell, A. M., Cotter, R. I. & Pardon, J. F. *Nucleic Acids Res.* **5**, 1571–1580 (1978).
18. Thoma, F., Koller, Th. & Klug, A. *J. Cell Biol.* **83**, 403–427 (1979).
19. Olins, D. E. & Olins, A. L. *J. Cell Biol.* **53**, 715–736 (1972).
20. Baudy, P. & Bram, S. *Nucleic Acids Res.* **6**, 1721–1727 (1979).
21. Bram, S. & Beeman, W. W. *J. molec. Biol.* **55**, 311–324 (1971).
22. Franks, A. *Proc. phys. Soc.* **B68**, 1054–1064 (1955).
23. Morimoto, H. & Uyeda, R. *Acta crystallogr.* **16**, 1107–1119 (1963).

Solid-like behaviour of unsheared sickle haemoglobin gels and the effects of shear

Robin W. Briehl

Departments of Physiology and Biochemistry, Albert Einstein College of Medicine, Bronx, New York 10461

Pathogenesis in sickle cell disease depends on the polymerization of deoxyhaemoglobin S into long fibres followed by 'gel' formation. The 'gelation' renders the affected erythrocytes less deformable than normal so that they obstruct the microvasculature and the 'gelation' process has been one of the targets for the development of therapeutic treatments of sickle cell disease. 'Gelation', however, acts through the rheological properties it induces and rheological abnormalities are therefore the immediate bases of pathogenesis. Although there has been very little study of the rheology of haemoglobin S haemolysates, the 'gels' are generally considered to be highly viscous and thixotropic on the basis of gross observation. Limited and generally qualitative observations show that high viscosity depends on deoxygenation^{1,2} and exhibits hysteresis in gelling/ungelling cycles¹; also that shearing accelerates gelation^{3,4} and can induce formation of fibre aggregates and crystals in suspension which, in contrast to the 'gel', are fluid⁵. Using transient and steady state methods it is shown here that unsheared sickle deoxyhaemoglobin preparations are solid-like, consistent with a gel-like nature, whereas shearing converts them to thixotropic viscous systems. These results underline the marked variation and thixotropy the system can undergo and may be relevant to the pathogenesis, clinical course and therapy of sickle cell disease.

Haemoglobin S solutions were prepared from homozygous haemolysates by chromatography on Whatman DE 52 (ref. 6), dialysis into 0.1 M potassium phosphate, pH 7.0, and deoxygenation at 0 °C under nitrogen only. These were transferred cold into a thermostatted Wells-Brookfield RVT Rheolog cone-plate microviscometer in a glove box kept free of oxygen by recirculation of 3% hydrogen in nitrogen over a palladium catalyst. The absence of oxygen was confirmed by (1) a methylene blue indicator, (2) polarographic analysis with a Clark electrode and (3) spectral examination of aliquots of haemoglobin removed from the viscometer. The viscometer cone angle (α) was 1.565° so that the speed range from 0.5 to 100 r.p.m. corresponded to shear rates from 1.92 to 384 per s. At 10 r.p.m. the relation between viscosity in centipoise, η_{cP} , and stress (σ) in dyn cm⁻² is $\eta_{cP} = 2.61\sigma$. The cone radius (R) was 2.4 cm. The

spring constant (k) for the coupling between the synchronous motor and the cone was 1,760 dyn cm. Shearing stress was observed on a digital readout and a continuous record of it and temperature, measured with a thermistor attached to the viscometer cup, was obtained with a Hewlett-Packard 7754A oscillographic recorder. Full scale stress was 245 dyn cm⁻², occurring at an angular lag of the cone behind the motor of 231°.

After the haemoglobin was introduced into the viscometer, shearing was begun at 10 r.p.m. and gelation was induced by a step increase in temperature. The half time of the step was about 2 s and, the last stages of warming being slower, final temperature was attained within 0.2 °C in about 2 min. (Confirmation that sample temperature increased at the same rate as that of the cup was obtained by observing the viscosity of a mixture of glycerol, 75% by weight in water, during a step increase in temperature.) Deoxyhaemoglobin S viscosity remained constant during the delay period incubation^{7,8}, after which it began to increase. For preparation of unsheared gels, the viscometer was turned off when viscosity reached 13 cP, about 4 times the solution value, and annealing under neither stress nor shear was then permitted for 30 min.

After each experimental procedure, the gel was melted by cooling to 1–2 °C for 1 h, the first 10 min in the absence of shear, followed by 40 min at 100 r.p.m. and then 10 min at 10 r.p.m. before the next induction of gelation by warming. Supportive evidence that this melting procedure fully reversed the gelation is provided by (1) the return of viscosity to the previous cold level, (2) constancy of viscosity towards the end of the 40 min period, (3) the reproducibility of the delay time and viscosity progress curve in the next induction of gelation and (4), the lack of change in observed properties of the gel when an experiment was repeated.

In Fig. 1a a gel incubated and annealed at 20 °C was subject to slowly increasing stress when the viscometer motor was turned on at 0.5 r.p.m. After the linear increase in stress, upon attaining full scale, the motor was stopped. Stress did not decay. In experiments of up to 10 days, no decay was seen (to within one part in 250). Employing the equation for viscous decay of stress in a cone-plate geometry $\sigma = \sigma_0 \exp(-3kat/2\pi R^3\eta)$ where t is time and η is viscosity (in poise), viscosity must be higher than 3×10^{10} cP. This very large value justifies designating the gel as solid-like rather than viscous. When, in some experiments, stress was relieved by rewinding the motor manually, re-application of stress produced identical results, confirming that the solid-like nature of the gel was not damaged.

During application of stress, the rate of increase was, within the limits of measurement (~1%), the same as that obtained (the

maximum rate) when similar stressing was carried out with the cone held fixed by water frozen in the viscometer. Therefore, the cone remained essentially immobile and the gel suffered little or no deformation; that is, because the rate of stress increase reflects deformation of the coupling spring, the rate of rotation of the cone, here essentially zero, can be calculated. A deviation of 1% in the rate of increase of stress from the maximum rate would correspond to an elastic shear modulus of 165 dyn cm^{-2} ; hence the modulus for the gel was equal to or greater than this.

At the three lower temperatures in Fig. 1a the gels showed yield values and peak stresses, both decreasing with decreasing temperature. Figure 1b shows a similar series of experiments, differing only in the time at which temperature was adjusted to the final level; the essential results are the same. Therefore, solid-like strength of gels, manifested in a yield stress, depends

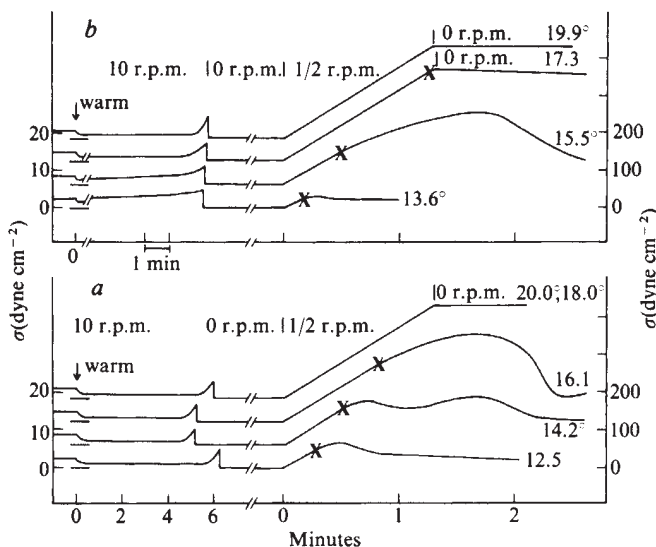


Fig. 1 *a*, Unsheared gels hold stress up to a yield stress, which depends on temperature. Five experiments were done sequentially on a sample 13.5 mM in haem at different temperatures. The ordinates represent shearing stress (σ) with a scale factor difference of 10 between left and right hand ordinates; the changes occur at the diagonal lines, at which time there is also a break in the time scale. The zero point on the ordinate scale applies to the experiment at 12.5°C ; the other curves are displaced upwards for clarity, with each baseline indicated. In each experiment a temperature jump (at the arrow) from 1°C to 20°C under shear at 10 r.p.m. resulted in a fall in viscosity (associated with warming). At the end of the delay period, stress and viscosity ($\eta_{\text{CP}} = 2.61\sigma$) rose until shearing was stopped. The temperature was then decreased in a few minutes from 20°C to that of the subsequent stressing experiment and the gel was annealed for 30 min under no stress or shear. Stress was then applied by running the synchronous motor at 0.5 r.p.m., stopping it only if stress reached full scale (as in the experiments at 20.0°C and 18.0°C). At 20.0°C and 18.0°C the curves were superimposable and both gels held stress at full scale with no decay. At the three lower temperatures the stresses at which the gels broke (as defined by a deviation of 5 dyne cm^{-2} from the line of maximal rate of stress increase) are indicated (X); these curves also showed maxima in stress as shearing at 0.5 r.p.m. was continued. *b*, in these experiments (on a different sample) gelation was induced by a temperature jump to a level which then remained unchanged through incubation, annealing and stressing. The delay times associated with the serially decreasing temperatures were 5.7, 11.0, 18.0 and 34.6 min. The viscosity progress curves differed and were more rapid at higher temperatures. The time scale is broken during incubation to make the starts of the 30 min annealing periods coincident. At 19.9° rotating the motor at 0.5 r.p.m. induced stress increase at the maximal rate. After the motor was stopped there was a very slow decay of stress. At 17.3°C a yield stress was attained just before full scale stress was attained; decay at 0 r.p.m. thereafter was more marked. At the two lower temperatures, successively smaller yield stresses and peak stresses occurred.

on temperature in the manner which would be expected from the endothermic nature of gelation^{1,9}. The energy of activation calculated from the delay times in Fig. 1b is 50 kcal mol^{-1} , neglecting any temperature-dependent effect of shearing.

Figure 2 defines a yield temperature and, reciprocal to the temperature dependence of yield stress in Fig. 1, shows that it depends on stress.

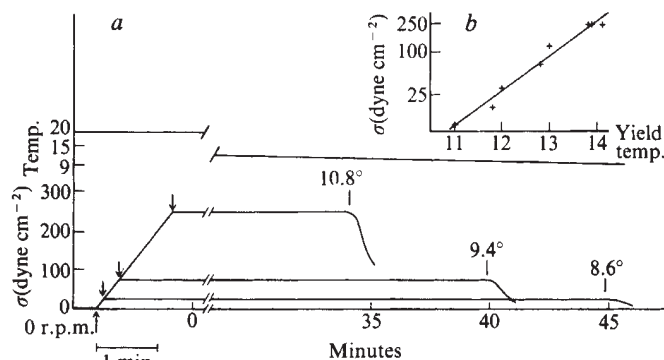


Fig. 2 Stress dependence of yield temperature. *a*, in separate experiments on a 14.0 mM sample, gels were formed and then annealed at 20° for 30 min as in Fig. 1a. Each gel was then stressed ($\uparrow$) by rotation of the motor at 0.5 r.p.m. until the desired stress was reached ($\downarrow$). At this stress, which did not decay, the gel was cooled slowly, beginning at time 0, until a rapid relaxation occurred, indicative of breaking of the gel. The yield temperatures thus defined, shown on each curve, decrease with decreasing applied stress. The time scale is broken at the diagonal lines. The temperature decrease shown is a superposition of the experiments and, because there were small variations in the rate of cooling, the times of breaking shown are approximate (within 1 min). The yield temperatures at the two higher stresses are each the average of two experiments, differing by 0.2°C or less. *b*, The dependence of yield temperature on stress (plotted logarithmically) is shown over a large, 40-fold, range of stress in a series of experiments on another sample, 13.7 mM in haem.

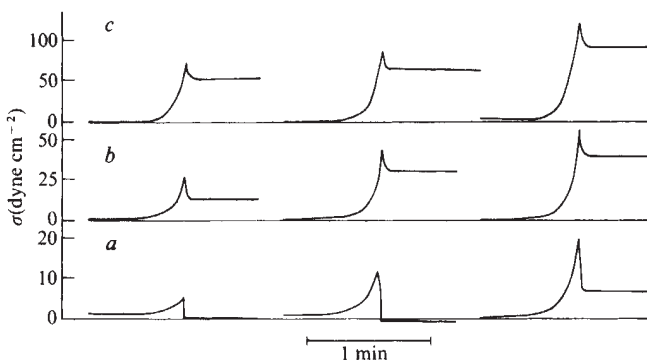


Fig. 3 Solid-like behaviour of gels during the growth stage of gelation. Nine experiments on a 13.4 mM sample at 26°C are shown. The delay times (defined here as the time required for viscosity to reach 13 cP) were all between 3.62 and 3.82 min. Shearing was carried out at 10 r.p.m. until a desired viscosity, from 13 cP in the first experiment in *a* to 320 cP in the last in *c*, was attained. In *a* full and rapid relaxation to zero stress occurred in the first experiment. In the second, spindle inertia induced a transient oscillation (off scale, below 0) in stress with a final constant value of -1.5 dyn cm^{-2} . In the third experiment the motor was stopped when viscosity was 56 cP. Full relaxation did not occur, as was also the case in all experiments in *b* and *c*. These gels held stresses without long term decay and were therefore solid-like rather than viscous. The rapid partial relaxation occurring immediately after peak viscosity is in whole or part an artefact associated with inertial properties of the cone spindle; it continued to rotate briefly after the motor had stopped and thus the transient rapid decay reflects this as well as (or instead of) any inability of the gel to hold the maximum stress attained. Thus, the final asymptotic stress is a lower limit on the stress the gel might hold.

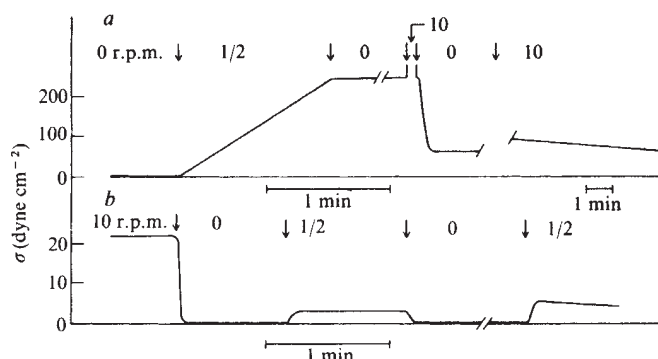


Fig. 4 Well sheared gels are viscous. *a* shows, as in Fig. 1, the stressing after annealing of a gel of 13.5 mM deoxyhaemoglobin S at 20 °C; full scale stress was held. Then the gel was sheared at 10 r.p.m. for 10 s (during which stress was off scale), resulting in partial relaxation to an asymptotic stress of 64 dyn cm⁻², indicating viscoplastic behaviour. Fifteen minutes later (during 8 of which the gel was sheared at 10 r.p.m.) the gel showed a time-dependent thixotropic decay in viscosity under continuing shear at 10 r.p.m. After prolonged shear for 120 min at 10 r.p.m., the viscosity reached a final constant value, shown at the start of *b*. Stopping the viscometer then resulted in rapid relaxation to zero stress. Under stressing at 0.5 r.p.m. the gel failed to hold any significant stress, a constant level reflecting viscosity only being attained. On stopping the motor, relaxation was again complete. After 30 min of annealing under no shear, the final part of *b* shows that rotation of the motor at 0.5 r.p.m. resulted in a peak in stress higher than that in the previous stressing, followed by a fall to a level dictated by viscosity, also higher than previously, indicating that some reannealing occurred.

In Fig. 3 shearing was continued after the delay period into the stage of fibre growth⁸ to different levels of viscosity. When shearing was stopped after viscosity had reached about 50 cP or more little or no relaxation occurred and the gels held large stresses, showing that the rheological behaviour was essentially solid-like rather than viscous.

By contrast, Fig. 4 shows that well sheared gels held no measurable stresses and were therefore purely viscous. Also, (apparent) viscosity decreased with time under shear and, after a period of no shear, reannealing occurred, demonstrating the presence of viscous thixotropy. Also in Fig. 4, gels subject to brief shear were viscoplastic, manifesting viscous decay to a non-zero asymptotic stress.

These results show that unsheared gels of haemoglobin S are solid-like, well sheared gels are purely viscous, and intermediate, viscoplastic conditions also occur. Also, thixotropy exists in the sense of conversion between solid-like and viscous behaviour as well as in the more usual sense of shear and time dependence of viscosity.

This observation of solid-like behaviour with a plastic yield stress provides justification for the designation 'gel', applied previously even though the system was generally described as 'highly viscous'.

The existence of solid-like properties may have a bearing on pathogenesis in sickle cell disease. An erythrocyte containing a purely viscous interior would deform to an extent dependent on the time integral of stress and thus might eventually pass the microvasculature. On the other hand, a solid-like erythrocyte could not deform unless a critical yield stress were attained. Because shearing history governs the solid-like or viscous nature of the gel, it may also govern the pathogenic potential of each erythrocyte. Whether shear is a favourable or unfavourable factor, however, depends on its effect on gel rheology and also on the fact that it accelerates the delay stage of gelation^{3,4}.

Generally, these observations show that gel rheology is highly modifiable even in the absence of alteration of the thermodynamics and kinetics of gelation, and thus that pathogenesis in this highly variable disease may be influenced by the highly variable rheology of gels.

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1. Allison, A. C. *Biochem. J.* **65**, 212-219 (1957).
2. Chien, S., Usami, S., Jan, K.-m., Smith, J. A. & Bertles, J. F. in *Proc. Symp. on Molecular and Cellular Aspects of Sickle Cell Disease* (eds Hercules, J. I., Cottam G. L., Waterman, M. R. & Schechter, A. N.) 277-300 (DHEW Publication No. (NIH) 76-1007, 1976).
3. Harris, J. W. & Bensusan, H. B., *J. Lab. clin. Med.* **86**, 564-575 (1975).
4. Briehl, R. W. in *Symp. on the Molecular Basis of Haemoglobin Dysfunction* (ed. Sigler, P. B.) (Elsevier, Amsterdam, in the press).
5. Pumphrey, J. F. & Steinhardt, J. *Biochem. biophys. Res. Commun.* **69**, 99-105 (1976); *J. molec. Biol.* **112**, 359-375 (1977).
6. Briehl, R. W. & Ewert, S. *J. molec. Biol.* **80**, 445-458 (1973); **89**, 759-766 (1974).
7. Malfa, R. & Steinhardt, J. *Biochem. biophys. Res. Commun.* **59**, 887-893 (1974).
8. Hofrichter, J., Ross, P. D. & Eaton, W. A. *Proc. natn. Acad. Sci. U.S.A.* **71**, 4864-4868 (1974).
9. Murayama, M. *Fedn Proc.* **15**, 318 (1956).

A variant surface glycoprotein of *Trypanosoma brucei* synthesized with a C-terminal hydrophobic 'tail' absent from purified glycoprotein

J. C. Boothroyd*, G. A. M. Cross*,
J. H. J. Hoeijmakers† & P. Borst†

* Wellcome Research Laboratories, Langley Court, Beckenham, Kent BR3 3BS, UK

† Universiteit van Amsterdam, Afdeling Medische Enzymologie, Laboratorium voor Biochemie, Jan Swammerdam Instituut, PO Box 60.000, 1005 GA, Amsterdam, The Netherlands

Sequential expression of variant surface glycoproteins (VSGs) enables the parasitic protozoan *Trypanosoma brucei* to evade the immune response of its mammalian hosts^{1,2}. Studies of several VSGs, which have been isolated as soluble molecules following disruption of cells in the absence of detergent, have indicated extensive amino acid diversity³⁻⁵ and the absence of a hydrophobic segment which might serve to anchor the carboxy terminus to the membrane⁵. The carboxy-terminal tryptic peptides of six VSGs have recently been characterized and shown to be glycosylated⁶. Three of these VSGs terminated with a glycosylated aspartate or asparagine residue (Asx), suggesting that the VSG was cleaved following synthesis and glycosylation and before characterization. We present here nucleotide sequence data which suggest that the primary translation product of one VSG gene contains a hydrophobic tail at the carboxy terminus which is not found on the isolated, mature glycoprotein⁶. The data also predict that the glycosylated residue is aspartic acid rather than the anticipated asparagine.

We have recently reported the synthesis and molecular cloning into the plasmid pBR322 of complementary DNA (cDNA) molecules corresponding to the VSGs of four variants of a single clone of *T. brucei*⁷. As extensive amino acid sequence data on one of these VSGs (VSG 117) already existed (G. Allen, personal communication), we have selected the corresponding recombinants (T-cV117·1-8) for further study. As purified VSG 117 terminates with a glycosylated Asx⁶, suggesting post-glycosylation proteolytic cleavage of a VSG precursor, we decided first to determine the nucleotide sequence corresponding to the 3' end of the mRNA, coding for the carboxy terminus of the protein.

A physical map of the cDNA insert which extended furthest in the 3' direction (T-cV117·8) and the protein region it covers is given in Fig. 1. Also shown are the regions where useful nucleotide sequence information was obtained. Figure 2 presents a representative sequencing gel corresponding to the carboxy-terminal portion of the protein. The complete nucleotide sequence of this region and the implied amino acid sequence are

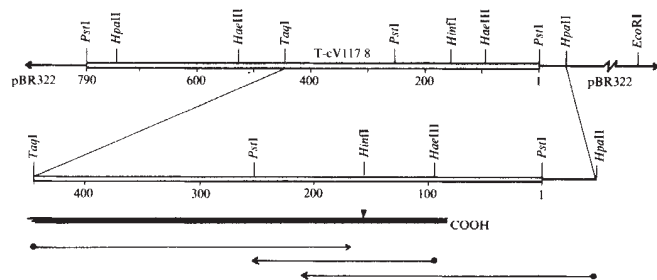


Fig. 1 Physical map of T-cv117.8 showing restriction endonuclease cutting sites. Distances are in base pairs from the end of the insert nearest the *EcoRI* site of pBR322. These data were obtained by standard mapping procedures and subsequently confirmed by the DNA sequence. Beneath the map are shown the region of the insert which codes for the putative primary translation product and the carboxy terminus of the mature VSG (▼). Below this are shown the sites which were [$5'$ - 32 P]-end-labelled (●) and the regions where useful sequence information was obtained (→).

given in Fig. 3. The fact that there is neither a poly(A) stretch before the dC tail in Fig. 3 nor the sequence (AATAAA) usually found about 25 nucleotides upstream of the site of poly(A) addition⁸ suggests that T-cv117.8 does not extend to the 3' end of the VSG 117 mRNA. This may be an artefact of the process by which these recombinants were constructed or selected⁷. The inferred amino acid sequence shown in Fig. 3 is in complete

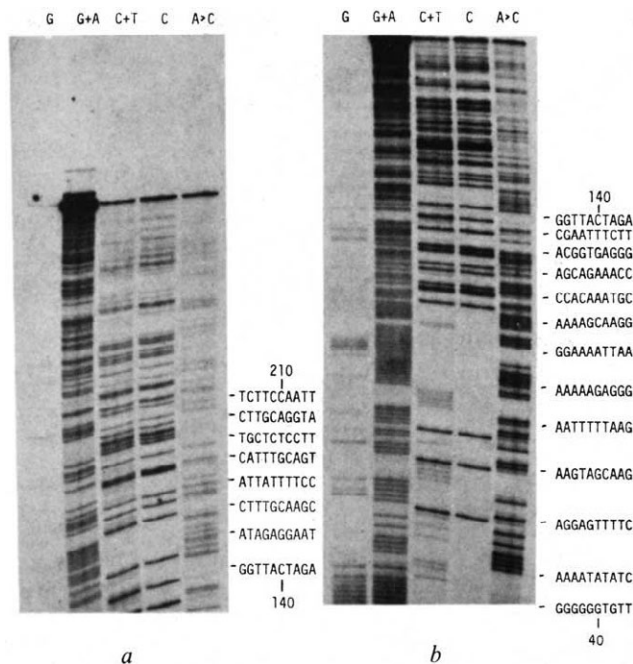


Fig. 2 Autoradiograph of a representative sequencing gel corresponding to the carboxy terminus of VSG 117. A *HpaII* fragment of DNA from T-cv117.8 was [$5'$ - 32 P]-end-labelled with polynucleotide kinase, recut with *TaqI* and purified on a 7% polyacrylamide gel essentially as described¹⁴ except that the eluted fragment was further purified by binding to a 1-ml column of diethylaminoethyl-cellulose followed by elution in 0.5 M NaCl. Base-specific modifications and cleavage were as described¹⁴, with minor alterations as recently recommended by the authors (A. Maxam and W. Gilbert, personal communication). Analysis was on a thin (0.4-mm) 8% polyacrylamide gel in 7 M urea run at high voltage (1.6–2.0 kV) to reduce secondary structure effects¹⁵. Aliquots of each sample were loaded on to different tracks of the gel at 0 (a) and 120 min (b), the gel being run for a total of 30 min. After cooling, the gel was fixed by immersing in 10% (v/v) acetic acid for 20 min, dried under vacuum with heat and autoradiographed at -70°C in a Kodak cassette containing a Dupont Cronex Xtra-life intensifying screen. The nucleotide sequence corresponding to the C-terminal region of the protein is given to one side. This sequence is of the noncoding strand, the nucleotides being numbered as in Fig. 1.

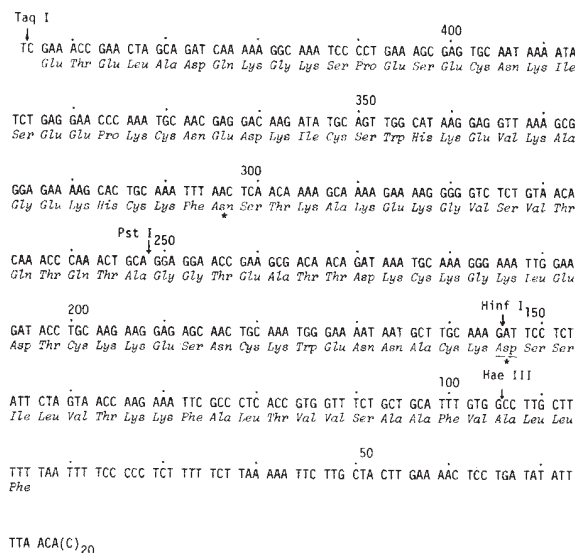


Fig. 3 Summary of nucleotide and protein sequence data. The nucleotide sequence of the coding strand between positions 445 and 1 (see Fig. 1) is given together with the implied amino acid sequence. Only one reading frame is open. The carboxy-terminal residue of the mature, isolated glycoprotein is underlined (see text). Glycosylated residues are marked with asterisks. (C)₂₀ indicates the dC-tail used to anneal the cDNA insert to the dG-tailed plasmid.

agreement with independently derived data from the purified glycoprotein (ref. 6 and G. Allen, personal communication) except that protein sequencing indicated the carboxy terminus to be exclusively glycosylated Asx at position 154, whereas the nucleotide sequence shows 23 sense codons beyond this position before a stop codon is reached. It seems, therefore, that at some time after translation of the polypeptide and before characterization of the VSG, a highly specific proteolytic cleavage has occurred 23 amino acids in from the carboxy terminus.

Although we have not directly examined the carboxy-terminal sequence of the primary mRNA translation product, it is a reasonable assumption that it contains the extension predicted by the DNA sequence. This would be consistent with our previous observation⁷ that 117 VSG mRNA translated *in vitro* yields a polypeptide which seems to be slightly larger on SDS-polyacrylamide gels than mature VSG isolated from trypanosomes, despite glycosylation of the latter which should increase its apparent molecular weight. The amino acid sequence towards the end of the predicted extension is extremely hydrophobic. We have been unable to define the role of the hydrophobic extension and the time at which it is cleaved. The fact that all six VSGs studied terminate with a glycosylated amino acid⁶ suggests cleavage may be a specific event common to VSG processing in *T. brucei*.

Hydrophobic carboxy termini have been reported in surface proteins of influenza virus⁹ and *Bacillus stearothermophilus*¹⁰ and may generally serve to anchor membrane-bound cell-surface proteins¹¹. Observations that carboxy-terminal glycopeptides of trypanosome VSGs carry an immunologically cross-reacting group⁶, which on living cells is not accessible to antibody¹², are consistent with the carboxy terminus being in close proximity to the surface membrane. If present in the VSG molecules forming the surface coat of living trypanosomes, the hydrophobic extension could serve a membrane-binding function. However, no precedent exists for the rapid and specific cleavage which would be required to explain the observation that mature VSG is released on cell disruption, even when several inhibitors of proteolysis are present (G.A.M.C., unpublished observations). The existence of such a mechanism for specific cleavage and release of VSG from the cell surface suggests a role in antigenic variation, possibly in allowing

shedding of VSG complexed with antibody. Clarification of whether the hydrophobic extension is present on VSG at the cell surface must await the results of further experiments. Alternatively, the hydrophobic extension could act as a signal sequence during processing and secretion of VSG and could have been removed before arrival at the cell surface (in a manner analogous to amino-terminal leader sequences¹¹). Comparison of the mature VSG amino terminus with the sequence inferred from the cDNA corresponding to the 5' end of the mRNA, however, indicates the presence of an amino-terminal extension similar to the signal sequences found on almost all secreted proteins¹¹ (J.C.B. and G. Allen, unpublished results).

Glycosylation of VSG 117 has been shown to occur at positions 304 and 154 (Fig. 3; ref. 6 and G. Allen, personal communication). The tripeptide Asn-Ser-Thr found at the first site (position 304) conforms to the general sequence Asn-Xxx-Ser/Thr thought to be a requirement for glycosylation at asparagine residues¹³. The other site, however, does not. Instead, the cDNA predicts the tripeptide sequence Asp-Ser-Ser, suggesting attachment of the carbohydrate moiety to

aspartate. To dismiss the possibility that this result was an artefact of the cDNA cloning procedure, a recombinant plasmid containing a genomic copy of the VSG 117 gene was analysed for the presence of the *Hinf*I cleavage site which is strictly dependent on the critical Asp codon (GATTC, positions 155–151, Fig. 3). The site was indeed present as defined by single and double digests with *Hinf*I and *Pst*I (J.C.B., unpublished results). It seems certain, therefore, that the primary translation product, at least, has Asp at position 154. We are now investigating whether the Asp is converted to Asn before glycosylation or is instead involved in a novel linkage to the carbohydrate. Similar analyses of cDNAs for other VSGs (now underway) should indicate whether the hydrophobic extension and glycosylated aspartic acid are universal features of the surface glycoproteins of *T.brucei*.

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1. Vickerman, K. *Nature* **273**, 613 (1978).
2. Cross, G. A. M. *Proc. R. Soc. B* **202**, 55 (1978).
3. Cross, G. A. M. *Parasitology* **71**, 393 (1975).
4. Bridgen, P. J., Cross, G. A. M. & Bridgen, J. *Nature* **263**, 613 (1976).
5. Johnson, J. G. & Cross, G. A. M. *Biochem. J.* **178**, 689 (1979).
6. Holder, A. A. & Cross, G. A. M. *Molec. biochem. Parasit.* (in the press).
7. Hoeijmakers, J. H. J., Borst, P., Van den Burg, J., Weissmann, C. & Cross, G. A. M. *Gene* **8**, 391 (1980).
8. Proudfoot, N. J. & Brownlee, G. G. *Nature* **263**, 211 (1976).
9. Porter, A. G. *et al.* *Nature* **282**, 471 (1979).
10. Waxman, D. J. & Strominger, J. L. *J. biol. Chem.* **254**, 4863 (1979).
11. Davis, B. D. & Tai, P.-C. *Nature* **283**, 433 (1980).
12. Barbet, A. F. & McGuire, T. C. *Proc. natn. Acad. Sci. U.S.A.* **75**, 1989 (1980).
13. Marshall, R. D. *Biochem. Soc. Symp.* **40**, 17 (1974).
14. Maxam, A. M. & Gilbert, W. *Proc. natn. Acad. Sci. U.S.A.* **74**, 560 (1977).
15. Sanger, F. & Coulson, A. R. *FEBS Lett.* **87**, 107 (1978).

Erratum

Figures 1, 2 and 3 were omitted from the letter 'Lower Cretaceous sediment from the East Antarctic continental shelf' by E. W. Domack *et al.* *Nature* **287**, 625–626.

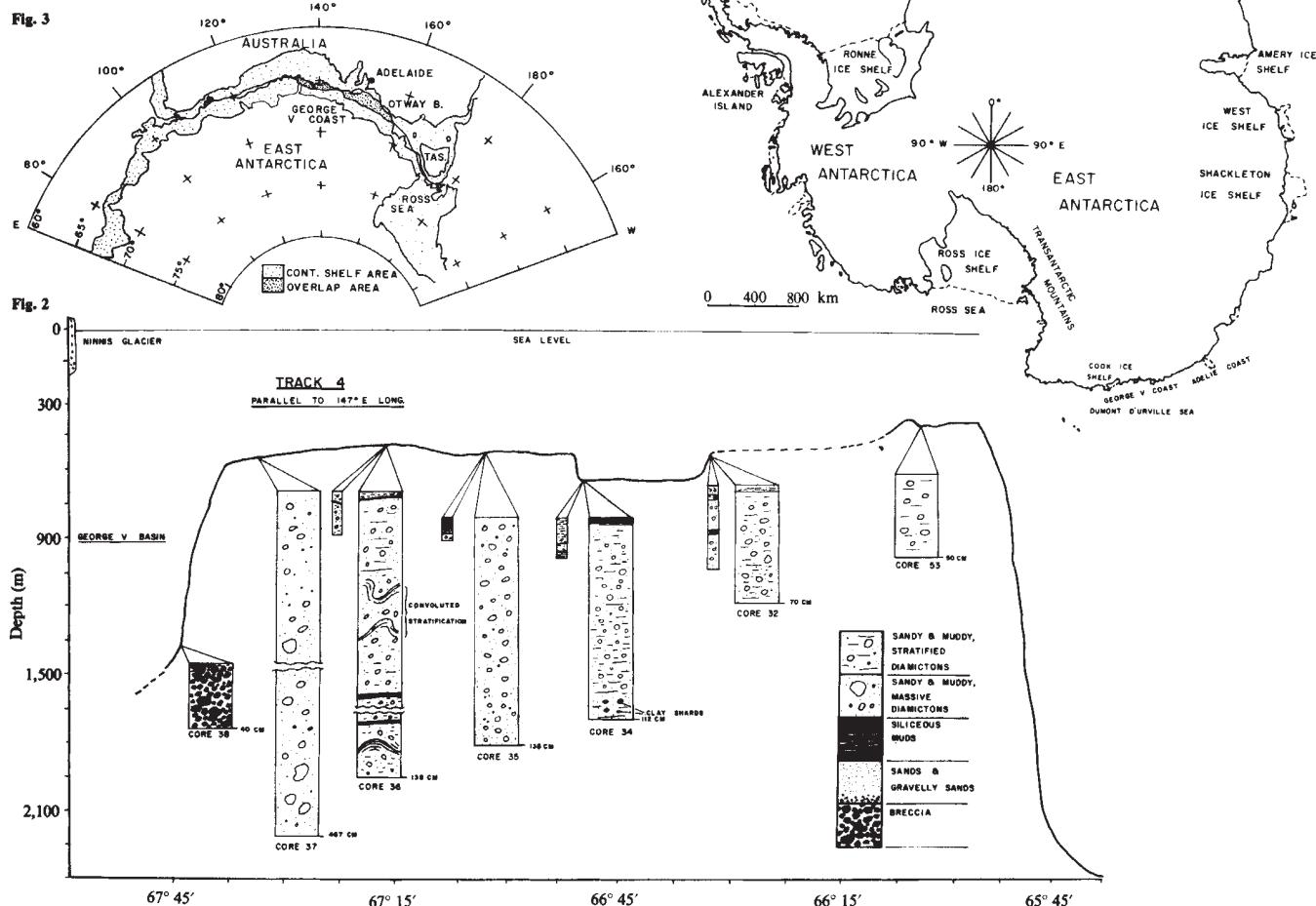


Fig. 1 Map of the Antarctic continent showing the locations of regions mentioned in the text.
Fig. 2 Profile of the continental shelf off the George V coast showing the relationship of core 38 to other sediments of the shelf (after ref. 7, Fig. 24).
Fig. 3 Morphological reconstruction of Australia and East Antarctica showing the positions of localities mentioned in the text (after ref. 9, Fig. 3).

BOOK REVIEWS

Science East and West: reflections of Peter Kapitza

Nevill Mott

PETER Leonidovich Kapitza was born on July 9, 1894, and began his scientific work in the Polytechnic Institute of St Petersburg, from which he graduated in 1918. In 1921 he was sent by the Soviet authorities to work under Lord Rutherford in Cambridge, where he stayed until 1934, the Royal Society Mond Laboratory being built especially for his work on the development and effects of high magnetic fields and low temperatures. During this time he retained his Soviet citizenship, returning to Russia from time to time and telling his friends that he thought he was the only man of his country with a passport endorsed for unlimited journeys. However, in 1934, while attending a conference in Leningrad to celebrate the one-hundredth anniversary of the birth of Mendeleev, he was told that he could not return to Britain, and would have to continue his work in the Soviet Union. I was at the same conference, and remember the impression that Kapitza's Rover car, driven from Bergen round the gulf of Finland, made among the few official cars in the streets of Leningrad. In Russia he continued work in low-temperature physics, for which he was awarded a Nobel prize in 1978; after the War he was accused of giving wrong advice about oxygen production and was excluded from his laboratory for several years; rehabilitated, he turned to plasma physics (not nuclear energy as is sometimes thought), and in the past few years has been able to travel abroad.

This book, translated from the Russian, contains a collection of articles and addresses delivered by Kapitza during the past 50 years. Many have appeared elsewhere, for instance "Recollections of Lord Rutherford" and a biographical memoir of L.D. Landau published by the Royal Society, of which Kapitza is a Fellow. His reverence for and gratitude to Rutherford are well known; he writes:

... in the year that Rutherford died there disappeared forever the happy days of free scientific work which gave us such delight in our youth. Science has lost her freedom. Science has become a productive force. ... rich but enslaved, part of her veiled in secrecy.

Sir Nevill Mott is Emeritus Professor of Physics at Cambridge University; he knew Kapitza well between 1930 and 1934.

Experiment, Theory, Practice: Articles and Addresses of P.L. Kapitza. Boston Studies in the Philosophy of Science, Vol.46. Pp.429. (Reidel: 1980.) Hardback Dfl.90, \$47.35; paperback Dfl.28, \$14.75.

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Peter Kapitza, July 1973

He writes, too, a moving appreciation of Paul Langevin, and of other figures from the past. From the past few years, when he could travel, come addresses to international bodies, such as one on the impact of modern scientific ideas on society to the Unesco symposium on the centenary of Albert Einstein. A few sentences will reflect his views about the way society is going in the developed countries of East and West alike.

The state compels a person to abide by its laws, and society compels him to live in accordance with his traditions. After a certain degree of well-being has been reached people begin to lose their liberty as their prosperity increases, and already the freedom of the individual in the developed countries is extremely limited. Modern society strives to organise people's lives in such a way that they will have an impression of freedom despite their lack of it. This is achieved by means of propa-

ganda and by turning people's interest towards sport, sex and entertainment so as to distract them from reality. Such were the principles on which society was organised in Aldous Huxley's *Brave New World*.

Kapitza seems to feel that it is not so very different in East and West. And in view of this, perhaps the most interesting parts of the book for the Western reader are his addresses to the Presidium of the Academy of Sciences of the USSR, for instance one in 1964, "The Effectiveness of Scientific Work". Here perhaps East and West do not appear so equal. He writes, in criticizing the situation in his own country:

An important factor in morale is to take part in scientific life abroad — at conferences and similar gatherings. As yet we take little part in them. Our delegates are still only one quarter or one fifth as many as those of the USA or other countries [are they now as many?] and often the delegates are selected by bureaucratic methods. . .

And in the same article, he writes that now nearly all those who should be leaders of science spend their time on committees, how every rouble must be accounted for even though money for research is not lacking. A director

cannot dismiss anyone, nor can he provide any incentive. The fettering of the Director, his restricted role in this important matter, is one of the reasons for the low efficiency of our institutes.

The book reveals Peter Kapitza's qualities that all his friends know well — his enormous courage, irrepressible vitality and humour. On humour, writing about theory and experiment, he quotes from the novel, *Gentlemen Prefer Blondes*, "that classical American work. . . 'Love is a good thing but a golden bracelet is forever'." His memory plays him wrong (or does he, as seems hardly credible, quote from a Russian translation?). What Anita Loos wrote in 1925 was "kissing your hand may make you feel very, very good but . . .". However, the point that Kapitza makes is that "theory is a good thing but a good experiment lasts for ever". Rutherford would have agreed, with the last part at any rate.

Models and mergers in mathematical ecology

Oscar Kempthorne

Theory of Population Genetics and Evolutionary Ecology: An Introduction. By J. Roughgarden. Pp.576. (Collier-Macmillan: 1980.) \$24.95, £15.95.

THIS large book is an attempt to present the basic ideation behind two fields of biology — population genetics and ecology — and to initiate the merger between the two which has long been overdue. Population genetics had a pre-Mendelian start with Galton, but, of course, it was the discovery of the Mendelian processes that led to the useful application of concepts of mathematical probability to the subject. Over the same period that Fisher, Wright and Haldane were formulating mathematical theories of the genetics of populations, workers such as Kostitzin, Lotka, Volterra and Gause were developing ideas about the dynamics of populations. In writing this book, Roughgarden is following the tradition of Haldane, Fisher and Norton who took the initial steps in combining the two streams of thought.

Roughgarden's purpose is to formulate mathematical models that incorporate aspects of birth, ageing and death, and to establish the consequences of such models. The mathematics involved in doing this become rather complex, but there is no doubt that this is one of the necessary approaches to a greater understanding of many biological processes. The result of this is that it is now common — indeed necessary — for departments of biology to include members of staff who have had a rigorous training in both biology and mathematics. Roughgarden is obviously an exemplar; he has made a fine effort to give an overall picture of a complicated field.

His book consists of five parts. The first is concerned with the basis of the subject, and in it he develops the elementary theory of a simple infinite Mendelian population with a two-stage life. Individuals contribute according to genotype (but not sex) to male and female genetic pools, new-borns arising by the random union of gametes. This leads into what Roughgarden calls "Wright's adaptive topography" (an unfortunate term), with the correct (but misleading) result that such a population will move towards maximum mean fitness. The rest of the opening part of the book is taken up with considerations of the fundamental theorem of natural selection for asexual populations — which has no genetic content at all — genetic drift (for a diallelic locus) and the neutrality controversy. This last chapter provides a good introduction to a somewhat obscure area.

I had anticipated that Part 2 would include detailed discussion of complex genetic systems. In fact, Roughgarden considers only models based on variation at

a multi-allelic single locus and two biallelic loci. Yet, even in this relatively simple case, what is really going on is far from clear (see Fig. 8.8, for example). This is followed by a rather curious chapter on natural selection and quantitative inheritance; the first half is purely biometrical and not Mendelian, the second does incorporate elements of simple Mendelism. Part 2 concludes with a consideration of Fisher's fundamental theorem — which is not at all as fundamental as Fisher thought — and a chapter on non-random mating, a reasonable introduction to inbreeding and assortative mating.

The next section of the book was, to me at least, the most disappointing. It deals with evolution in general, and in spatially varying and temporally varying environments, and with the development of altruism; few results having general implications have come from recent work in these areas.

Once again, in the first five chapters of Part 4, one soon gets involved in difficult integral equations. Here, Roughgarden considers the dynamics of an asexual population in what is a neat, but perhaps oversimplified, presentation. Finally, in this part of the book, stochastic environments are treated; here, it is a great surprise to find the use of Ito and Stratonovich stochastic calculus.

The final section of the book deals with

interacting populations. It starts with a consideration of competition between asexually reproducing populations and leads on to predator-prey models — a useful exposition. Then we are given chapters on coevolution (incorporating a single Mendelian locus and based mostly on Roughgarden's own recent work) and on niche theory and island biogeography.

My overall impression of the book is that it provides a useful but on occasions oversimplified picture of research in the field between 1910 and 1950. Its real value lies in that large portion which deals with more recent work, that undertaken over the past decade, and in this it will undoubtedly be of great value to specialists in mathematical ecology. A deep lacuna in the whole effort is the absence of real quantitative data and then the testing of models by reference to such data. Part of this is due to the immense difficulty of obtaining "hard" data on the dynamics of real ecological systems.

Theoretical ecology incorporating genetics is a young yet complicated area of biological enquiry. We must be grateful to Roughgarden for giving us a base from which to proceed. □

Oscar Kempthorne is Professor of Statistics and Distinguished Professor in Sciences and Humanities at Iowa State University, Ames, Iowa.

Thermodynamics and macromolecules

S. P. Spragg

An Introduction to Physical Properties of Large Molecules in Solution. By E. G. Richards. Pp.298. (Cambridge University Press: 1980.) Hardback £18, \$29.50; paperback £5.95, \$9.95.

TO MANY teachers of biophysical chemistry it seems strange to note that over the past two decades instruction on the subject of macromolecules has progressed to a position of marked contrasts. On the one hand, structural descriptions of macromolecules are based on accurate methods of X-ray crystallography, so students persevere in understanding details of this technique. On the other, emphasis in the teaching of the properties of macromolecular solutions has moved slowly away from exact thermodynamic interpretations and so student interest in the fundamental understanding of thermodynamics has waned. It is a pity that some recipe cannot be found which convinces students that the three laws of thermodynamics provide a thread for connecting

many phenomena associated with macromolecular solutions.

The present book contains little philosophical discussion along these lines but it is particularly good at referring proofs back to the fundamental laws of thermodynamics without stating the laws as a starting point. The contents range over configurational statistics of polymers, helix-coil transitions and hydrodynamic properties of macromolecules, as well as providing sound introductions to molecular interactions of neutral molecules and polyampholytes. The lack of experimental results which direct the students' attention to the phenomena under discussion means that the book must be supplemented through lectures; for example, experimental results from the melting curves of nucleic acids would have formed a useful start to the discussion on helix-coil transitions. Further examples will come to the mind of other readers, but this criticism is minor and only serves to show that this book is mainly concerned

with theoretical treatments of problems.

It was particularly pleasing to find a chapter devoted to scattering of electromagnetic radiation (written by S. D. Dover), since some understanding of this phenomenon at a fundamental level is becoming more important as laser technology expands the possibilities of measuring molecular properties on very short time scales.

The book will provide a useful reference text for undergraduates requiring guidance in the application of chemical thermo-

dynamics to describing macromolecular properties. Further, like Professor C. Tanford's well known text on a similar topic (*The Physical Chemistry of Macromolecules*; Wiley, 1961), the content is such that it will also be of value to postgraduates with an interest in the subject.

S. P. Spragg is Senior Lecturer in Physical Chemistry at the University of Birmingham, UK, with research interests in the solution properties of biological macromolecules.

New membranes for old

Harvey Lodish

Cell Membranes and Viral Envelopes. Edited by H. A. Blough and J. Tiffany. 2 volumes, pp.848. (Academic: 1980.) Both volumes £40, \$92.

MOST lipid-containing animal viruses bud from either the plasma membrane or internal membranes of infected cells. Much of our knowledge of the structure and assembly of membrane components has come from the use of defined virus model systems. These two volumes represent a noble and needed attempt to bring together reviews on the structure and function of both viral and cellular membranes and their constituents. There are chapters, written by recognized experts, on the basic physical and microscopic techniques used in membrane research, and on the biochemistry of viral and cellular membrane lipids, proteins and carbohydrates. In addition there are contributions on the structure and assembly of particular groups of viruses — influenza virus, retroviruses, Herpes virus and rhabdoviruses — though, oddly, none on the paramyxoviruses or arboviruses.

Most of the contributions to these volumes were written in 1974 and 1975. The end result is that the coverage of publications during the past five years is very erratic. Incredibly, the two chapters written by the editors contain references to very few articles published after 1976. What is worse, the editors apparently exercised little direction over the content of individual chapters, nor did they attempt to rewrite, up-date, or interrelate them. The predictable result is extensive redundancy of data and confusion between chapters.

As one example, the partial sequence of glycoporphin used in the chapter on the structure and function of membrane proteins is taken from the 1971 preliminary report; there is no reference to the complete sequence published in 1975 or to the 1978 revised sequence. Oddly, the complete sequence is contained in the following chapter, "Carbohydrates in the Cell

Membrane". Further, in no contribution is there mention of the techniques of fluorescence photobleach recovery, now probably the most widely used method for the determination of the extent and rate of lateral diffusion of cell surface components.

The chapter on carbohydrates of viral envelopes was written in 1974, and is very dated, but some of the more recent structural work is contained in the following chapter, "Glycosylation of Viral Envelope Components". None of the contributions discusses recent work on the importance of proteolytic cleavages in the maturation of viral glycoproteins, such as the influenza haemagglutinin HA or the paramyxovirus fusion protein F. The contribution "Synthesis of Viral Proteins" was also written in 1974-1975, before the site of synthesis of viral glycoproteins in the rough endoplasmic reticulum was known; this chapter, unfortunately, is now largely of historical interest only. Like several others, it contains a one-page addendum which mentions some published work up to the beginning of 1977, but there still remains an inexcusable three-year delay before even this was published.

Some of the individual reviews dealing with more recent developments are of high quality. Many others would have been quite useful had they been published within a year or so of writing, surely a more reasonable lag time. These include those on virus receptors; on influenza virus; on structure and on synthesis of membrane and viral lipids; and on membrane changes in virally transformed cells. The fields covered by this book are expanding very rapidly; as a consequence, these reviews will not be of much value to the contemporary student or researcher. It is indeed unfortunate that the considerable effort expended in writing this book has been largely wasted, as current reviews of these topics would be valuable.

Harvey Lodish is Professor of Biology at the Massachusetts Institute of Technology, Cambridge, Massachusetts.

Geothermal maps

Philip England

Atlas of Subsurface Temperatures in the European Community. Compiled by R. Haenel with co-authors. Large format, pp.36+43 maps. Commission of the European Communities/Th. Schäfer: Hannover, 1980.) DM 120.

THE production of subsurface temperature maps for an area the size of the European Economic Community is an undertaking that requires a degree of faith. If it can be assumed that the available, sparsely distributed, temperature and thermal conductivity data adequately characterize the near-surface heat flow of the region (and that this is not perturbed by fluid motion), then it requires only a knowledge of the subsurface geology, and of the thermal conductivity and heat generation of the major lithologies, to extrapolate measured temperatures outward and downwards to make the kind of map this book contains.

These maps are each about 40 cm × 50 cm in size. Seven are 1:5,000,000 maps of subsurface temperature distributions beneath Europe at depths between 500 m and 5 km; six show subsurface temperatures at 1 km for the countries of the EEC; and some 20, with scales of between

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Leo Szilard: *His Version of the Facts*, edited by Spencer R. Weart and Gertrud Weiss Szilard (for review see *Nature* 278, 285; 1979) has been re-issued in paperback by the MIT Press, price \$7.50, £4.95.

1:500,000 and 1:75,000, indicate subsurface temperatures in areas of geothermal interest where the density of subsurface temperature measurements is highest.

Compiling these maps has clearly involved assembling vast amounts of subsurface temperature data (otherwise not readily accessible), and where the density of these observations is high — in the upper kilometre or so of regions of known geothermal potential — the maps may be regarded as reasonably accurate summaries of the available information. Their use is limited, though, when the density of observation is low and when extrapolation over depths of several kilometres is required.

Anyone contemplating buying this book should look first at Plate 2, which shows the density of the available heat flow information in the EEC, and at Plate 39 which indicates the amount of information provided by boreholes. Next they should read, in any one of six languages, the four-page introductory text which gives a brief exposition of how the maps were compiled and of the pitfalls involved in making the kind of extrapolation outlined above. (One point not dealt with adequately is the error involved in assigning values of conductivity and heat production when extrapolating temperatures downwards; the compiler's assumption that the likely uncertainty in these parameters is 10–15% is a wildly optimistic one, and leads him to place misleadingly small error bars on the temperature estimates that he makes.)

Having taken these steps one should be convinced of the difficulties involved in predicting subsurface temperatures from the information available at present, and hence that this book is of little use as a predictor of geothermal resources. The book's remaining attribute, on which it should be judged, lies in the compilation of information that it represents. It is a shame, therefore, that the introduction is so cursory, that so little indication is given of the reliability of the data, and that it was thought necessary to present the information in this elaborate guise.

Philip England is a Research Fellow in the Department of Earth Sciences, Bullard Laboratories, University of Cambridge.

Herbal facsimiles

Sandra Raphael

Herbarium Apulei Platonici (Vol.I) and *Herbolario Volgare* (Vol.II). Facsimile reprints. Introductions by Erminio Caprotti and William T. Stearn. Pp.CXXII+566. (Edizioni Polifilo: Milan, 1979.) Both volumes L.85,000.

A NEW pair of facsimiles reprints the first Latin and the first vernacular herbals published in Italy, the former in 1481, the latter in 1522. The 1481 book, probably the earliest illustrated herbal printed, is the *Herbarium* of Pseudo-Apuleius, a Graeco-Roman compilation with mediaeval trimmings, surviving in a multitude of manuscripts that indicate its status as a standard source of information on medicinal plants. The crude, diagrammatic woodcuts can have given little help in the identification of the plants described.

The 1522 *Herbolario Volgare* took both

its text and its illustrations from the *Latin Herbarius* printed by Peter Schöffer, Gutenberg's successor, in 1484. The text is another miscellany from an unknown hand, but its pictures are a decorative improvement on those of the Apuleius. The Iringo (sea-holly) is shown here, a plant whose candied roots were regarded as an aphrodisiac as late as the seventeenth century.

Professor Stearn's introductory essay appears in both Italian and English, and the books are fine examples of the exquisite printing of Mardersteig's Stamperia Valdona.

Sandra Raphael is a Senior Editor in the Dictionary Department of Oxford University Press. Her book on illustrated herbals, title?, written in collaboration with Wilfrid Blunt, was published late in 1979.



IRINGO LXXVI

¶ Dello Iringo. Cap.LXXVI.
 Lo iringo sic caldo & humido nel primo grado: Et la sua humidita e maggiore della sua calidita: & quelli che usano la radice condita cō melle moltiplica il sperma: & fa al coito: & allo drizare della uerga: & fa bon nutrimento: Dice serapione & Auicena: Et le radice de iringo cō uno pocho de specie aromatiche si mágiano: ouero con melle o zucharo & cinamomo si acóciano: Lo iringo sic una sorte de spina le foglie delloquale si agionge